A metabarcoding approach to assess the diet of lacertid lizards from the High Atlas Mountains

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2017

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

Acknowledgements

These two years made me grow in such a scientific and personal way that I could have never imagined. All the challenges I had to face made me learn in the most interesting and fun way. It has been a life lesson. I am truly grateful to everyone that helped me with the smallest thing from the moment I started until now. It was such a pleasure for me to do this thesis.

First and most important of all, I have to thank to my supervisors for giving me the opportunity to work with them. I feel really lucky for having the best of three completely different personalities working with me. James, thank you for teaching me how to make science (even though I am only starting!), and thank you for always believing in what I was capable of and for making me believe too. Thank you for your patience when my brain started to freeze and for all the jokes, specially about my bad luck! And, of course, thank you for Morocco. Raquel, thank you for living this thesis so intensively as I did. Your enthusiastic motivation made me appreciate this thesis more and more. Thank you for always being there and help me in every possible way. You were tireless from the beginning to the end. Ana, you know me since I was still a baby in science and you helped me growing till this step with the most helpful advices and conversations. Thank you for all your passionate ideas and everything you taught me with the biggest patience ever. Thank you for your kindness and for your constant smiling. To you three, thank you for all the advices, for believing in me, and for being more than my supervisors.

Thank you to everyone that helped me in some way at CIBIO, specially the Ecolab. Thank you to Simon Jarman and all the Environmental Metagenomics group for the important recommendations and discussions throughout the course of this project. To my masters' colleagues with whom I spent most of my days, thank you for going through all this with me.

Thank you to my housemates and specially thank you to my friends from Barcelos: Du, Melo, Diana, Dani, Nela and Moura. For being by my side no matter what and for all the moments you gave me the strength to believe in myself. Thank you to Dihem, who was with me during the worst and last months. For always bringing his joy, love, good energies, singing and dancing around the house, and for all the times he cooked for me.

Thank you to Célia and Dani, who were so important during these two years. For all the support and conversations, for coming to Porto just to spend 10 minutes with me, for encouraging me to never give up from what I really love and for spending some of the greatest moments of my life with me.

Thank you to Dan, for being my family during this entire year and for taking care of me. For the best and worst moments always by my side, for helping me growth as a person, friend and scientist. For all the pancakes, tea, gin, soap operas, concerts, the most random conversations and the most important conversations. Thank you for the unconditional help in everything since we met in the lab. Thank you for all the patience and for helping me getting through everything I had to face in the last times. Thank you for this friendship and for making me laugh to die with the most ridiculous things!

Lastly, thank you to the most important people in my life: my family. Mom and dad, thank you for your constant support throughout my life and for the person I am now. Thank you for encouraging me to always do what I love, no matter what. Inês, thank you for your craziness, all the pointless arguments, all the selfies, and all the songs. Thank you for loving me more than anyone, and the most important thing: obrigada por bateres maleiro comigo!

Obrigada!

Abstract

Dietary analyses are a fundamental tool in helping to define ecological niches and trophic relationships. Closely related species sharing the same habitat might reduce competition by exploiting different resources, which might be reflected into different microhabitat use and foraging modes. Although lizards are mainly opportunistic feeders, some foraging strategies might be more advantageous than others, shaping the types of consumed prey. Two extreme foraging modes are recognized: sit and wait foraging and active foraging. Sit and wait foragers wait for prey to approach from a stationary place and quickly attack them, and thus are expected to find more mobile prey and have a less diverse diet. On the other hand, active foragers are more likely to find sedentary, clumped and unpredictably distributed prey as they look actively for food. These distinct foraging strategies are also dependent on different morphological features, and acceleration and speed are two key features since the way lizards move in the field influences both exposure to predators and the ability of catching prey. Another important feature is dorsal coloration, where cryptic spotted patterns are more common in sit and wait predators, whereas active foragers are more likely to present striped patterns creating a 'motion dazzle' phenomenon when moving. *Scelarcis perspicillata* is a species complex of lacertid lizards endemic to Morocco. Two forms are known to live in strict syntopy in a specific location, Taza - *S. p. chabanaudi* and *S. p. pellegrini*. In this thesis, we studied the dietary habits of these two forms, using a DNA metabarcoding approach, in order to understand to which extent they can be segregating food niches to reduce intraspecific competition. Based on their dorsal pigmentation patterns, and on previous studies regarding escape tactics and diet, we expected *S. p. chabanaudi* to be a sit and wait forager and *S. p. pellegrini* an active forager. Only one dietary study was previously performed for this species in Taza, about on decade ago, based on microscopic examination of faeces. My results confirmed the differences previously found between the two forms. However, we concluded that although the diet diversity of the two species conformed with the expectations regarding foraging modes, diet composition suggests both species are able to adopt both foraging strategies. Morocco harbours other endemic cryptic species complexes, such as the case of the lizard *Atlantolacerta andreanskyi*. The diet of this species was already assessed in the selected study area, Oukaïmeden, where a community study was performed more than a decade ago. My aim was to investigate diet partitioning between males and females, as well as ontogenetic differences. Although the results showed small differences between sexes, adults had a much more diverse diet than juveniles. Moreover, juveniles consumed a higher

proportion of soft bodied prey, which might be due to the longer handling time required to consume harder prey. In general, my research is in accordance with the previous one, with the additional detection of two prey items not described before.

To understand food web structures, dietary assessments were initially performed through microscopic observation of food remains in gut or faecal contents. However, this method requires taxonomic expertise and not always allow the detection of highly digested or degraded food items. Levered by the current taxonomic expertise shortage, DNA metabarcoding is revolutionizing dietary studies as it provides the possibility to quickly and effectively identify prey items using non-invasive sampling. This technique relies on the amplification of short barcodes through high-throughput sequencing and subsequent comparison with public taxonomic databases. Nevertheless, the accuracy of the results depends on several steps of the metabarcoding “pipeline” that can cause biases: from the marker choice, PCR conditions, the use of blocking primers, bioinformatic analysis, among others. The present diet study of the species from Taza and Oukaïmeden was based on the amplification of COI and 16 rRNA markers in a dual-indexing sequencing strategy.

Overall, the use of metabarcoding approaches revealed the existence of multiple technical factors that need to be considered in dietary studies in reptiles. One of them is the extreme importance of marker choice as the use of some markers might prevent the detection of some taxa. Moreover, this method had the drawback of only providing a list of the present species plus the limitation of not distinguish between the different life-stages of prey. On the other hand, greater diversity of prey was identified in *A. andreanskyi* than had been recorded with microscopy, and by being able to identify prey to the family level (rather than order) differences in the diet between males and females could be detected. We recognized that both the advantages and limitations can have huge implications when using dietary assessments to infer ecological characteristics of the lizards, and as all investigations, it demonstrates the advantages of using an integrative approach for a more complete assessment.

Keywords

Scelarcis perspicillata; *Atlantolacerta andreanskyi*; metagenomics; COI; 16S rRNA; foraging stategies; ontogeny; sexual dimorphism.

Resumo

Estudos sobre dieta são uma ferramenta fundamental para delinear nichos ecológicos e interações tróficas. Estima-se que espécies filogeneticamente próximas possam coexistir se explorarem diferentes recursos, o que por sua vez se reflete em diferentes usos do *microhabitat* e na adoção de estratégias de alimentação distintas. Embora se considere que a maior parte dos lagartos tenha uma alimentação oportunista, o consumo de certos tipos de presas pode ser mais vantajoso, moldando a preferência alimentar. São reconhecidos dois modos distintos de procura de alimento: *sit and wait* e *active forager*. É esperado que os animais que adotam a primeira estratégia aguardem que as presas se aproximem para depois rapidamente atacá-las, e por isso é mais provável que encontrem presas mais móveis e que tenham uma dieta menos variada. Por outro lado, os *active foragers* têm maior probabilidade de encontrar presas mais sedentárias, agregadas e distribuídas imprevisivelmente. Estas diferentes estratégias de procura de alimento dependem também de diferentes características morfológicas, sendo que a aceleração e a velocidade são muito importantes, uma vez que a forma como os lagartos se movimentam influencia tanto a sua exposição aos predadores, como a sua capacidade de captura de presas. Outra característica importante é a coloração dorsal, em que padrões com manchas ou pintas são mais comuns entre predadores *sit and wait*, enquanto que os *active foragers* são mais propensos a apresentar padrões com riscas, confundindo visualmente os predadores quando se movem. *Scelarcis perspicillata* constitui um complexo de espécies de lacertídeos endémicos de Marrocos. Duas subespécies vivem em sintopia em Taza, Marrocos: *S. p. chabanaudi* e *S. p. pellegrini*. Nesta tese foram estudados os hábitos alimentares destas duas subespécies usando *DNA metabarcoding*, com o intuito de compreender em que medida poderá estar a ocorrer segregação de nichos como forma de adaptação para reduzir competição intraespecífica. Com base nos seus padrões de coloração e em estudos anteriores sobre táticas de fuga e dieta, admitimos a hipótese de *S. p. chabanaudi* ser *sit and wait forager* e *S. p. pellegrini* um *active forager*. Apenas um estudo sobre a dieta desta espécie foi anteriormente realizado, através da observação microscópica de fezes. Os resultados do presente estudo confirmaram as diferenças encontradas anteriormente entre as duas subespécies. No entanto, foi possível concluir que, embora a diversidade da dieta das duas subespécies esteja em conformidade com as expectativas em relação às estratégias de procura de alimento, é de notar que a sua composição da dieta sugere que ambas são capazes de adotar ambas as estratégias.

Marrocos constitui habitat para outros complexos de espécies crípticas endémicas, como é o caso do lagarto *Atlantolacerta andreanskyi*. A dieta desta espécie foi examinada apenas uma vez na mesma localidade analisada neste estudo, Oukaïmeden, há mais de uma década. Temos, então, como objetivo investigar a partição da dieta entre machos e fêmeas, assim como diferenças ontogenéticas. Embora os resultados não tenham permitido encontrar grandes diferenças entre sexos, os adultos mostraram ter uma dieta muito mais diversificada do que os juvenis. Para além disso, os juvenis consumiram uma maior proporção de presas constituídas por tecidos macios ou moles, o que pode ser causado pela dificuldade em manipular presas rígidas por parte dos mesmos. No geral, este estudo está de acordo com a investigação anterior, sendo que permitiu a deteção de duas presas não detetadas anteriormente.

O estudo da estrutura de cadeias tróficas é tradicionalmente baseado na observação microscópica de restos alimentares presentes no estômago ou fezes dos animais. No entanto, este método requer elevados conhecimentos taxonómicos e nem sempre permite a deteção de presas altamente digeríveis ou degradadas. Promovido pela reconhecida perda de *expertise* taxonómico que se verifica atualmente, o *DNA metabarcoding* tem revolucionado estudos sobre dieta, uma vez que permite identificar rápida e efetivamente diferentes tipos de presas consumidas. Esta técnica baseia-se na sequenciação *high-throughput* de fragmentos de DNA que funcionam como *barcodes* e na sua posterior comparação com bases de dados taxonómicas públicas. No entanto, a precisão dos resultados depende das várias etapas do processo de *metabarcoding* que podem causar enviesamentos, tais como a escolha do marcador a amplificar, condições de PCR, a utilização de oligonucleótidos para bloquear a amplificação do predador, análise informática, entre outros. O presente estudo teve como base a amplificação dos genes COI e 16S rRNA, numa estratégia de sequenciação *dual-indexing*.

Em geral, o uso de *metabarcoding* revelou a existência de múltiplos fatores técnicos que necessitam de ser considerados num estudo sobre dieta em répteis. Um deles é a importância da escolha dos *primers* de forma a garantir a deteção de todos *taxa.* Além do mais, esta técnica apresenta a desvantagem de apenas conseguir identificar a lista das espécies presentes, sem conseguir distinguir entre os diferentes estádios de vida das presas, um aspeto importante da análise de dietas. Por outro lado, uma maior diversidade de presas foi identificada para o lagarto *A. andreanskyi*, comparativamente com o que tinha sido descrito anteriormente através da analise microscópica e, sendo capaz de identificar presas ao nível da família (ao invés da ordem), diferenças entre machos e fêmeas puderam ser detetadas. É possível concluir,

portanto, que tanto as vantagens como as limitações podem ter grandes implicações quando se depende de estudos em dieta para inferir caraterísticas ecológicas de lagartos, e que, como em todas as investigações, há uma maior vantagem em fazer uma abordagem integrativa de forma a obter um estudo mais completo.

Palavras-chave

Scelarcis perspicillata; *Atlantolacerta andreanskyi*; metagenómica; COI; 16S rRNA; estratégias de procura de alimento; ontogenia; dimorfismo sexual.

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List of Abbreviations

BOLD	Barcode of Life Data Systems
COI	Cytochrome C Oxidase Subunit I
DNA	Deoxyribonucleic Acid
IUCN	International Union for Conservation of Nature
m.a.s.l.	Meters above sea level
$MgCl_2$	Magnesium chloride
NCBI	National Center of Biotechnology Information
NGS	Next Generation Sequencing
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
rRNA	Ribosomal Ribonucleic Acid
SVL	Snout-Vent Length

1. Introduction

1.1. General introduction

The way living organisms are connected in the tree of life and the evolutionary basis of biodiversity are a result of the adaptation of different strategies for survival. One of those strategies is the partition of the available resources within ecological communities (Pianka, 1974). Given competitive exclusion, it is thought that closely related species are better able to coexist if they specialize and exploit different resources (Pianka, 1966), and consequently changes in their morphology, physiology and behaviour are promoted in order to exploit such resources more efficiently (Verwaijen et al., 2002). Therefore, differences in microhabitat use or foraging strategies are expected to translate into different prey selection (Carretero et al., 2006).

Dietary analyses are fundamental for understanding ecological relationships among organisms, and are an important tool in helping to define ecological niches and trophic relationships. The diet of an organism is delimited by the availability of resources, but also by a wide range of intrinsic factors such as body size (Reading & Jofré, 2013), gender (Perry, 1996), age (Paulissen, 1987) or morphology (Scali et al., 2016). Other factors, such as population density, competition or ecology (Carretero et al., 2006) are also key aspects regarding the diet.

Although it is thought lizards are mainly opportunistic insectivorous feeders (Díaz, & Carrascal, 1990), some types of foraging strategies might be more advantageous in certain circumstances than others, ultimately shaping the types of prey items consumed. Lizards spend much of their active time foraging, and thus, their detectability to predators can be determined by the type of movement used in the foraging behaviour (Halperin et al., 2016). Pianka (1966) recognized two extreme foraging strategies: sit-and-wait foraging and active foraging, which can be associated with species-specific behavioural, physiological, and morphological traits (“the syndrome hypothesis”, McLaughlin, 1989). Sit-and-wait foragers chase for prey from a stationary place and quickly attack the prey when it enters their field of perception, whereas active foragers move more frequently throughout extensive areas looking actively for prey (Verwaijen & Van Damme, 2007).

Following these two contrasting behaviours, active foraging lizards are more likely to eat sedentary, clumped and unpredictably distributed preys such as insect larvae or termites, while sit-and-wait predators tend to find more active and mobile prey which are

expected to be larger and more profitable as they adopt a movement minimization strategy to avoid predation (Huey & Pianka, 1981; Verwaijen & Van Damme, 2007). Morphological features affecting locomotor ability are also predicted to be influenced by foraging style, and thus actively foraging lizards in particular should have adaptations to reduce the energetic cost of their constant movement (Verwaijen & Van Damme, 2007). Moreover, differences in head and body size, through their effect on bite force capacity, may also affect prey selection (Verwaijen et al., 2002) leading to space and niche segregation.

Several species endemic to Morocco have shown to constitute a species complex, such as the lizard *Scelarcis perspicillata* (Harris et al., 2003; Perera et al., 2007). Two forms of this lacertid are known to live in syntopy in Taza: *S. p. pellegrini* and *S. p. chabanaudi*. A previous study on their diet in this locality (Perera et al., 2006) revealed more differences between these two forms when living in syntopy than in allopatry. Therefore, we expect them to have different ecologies such as foraging modes and dietary preferences, in order to reduce intraspecific competition. Moreover, the lizard *Atlantolacerta andreanskyi*, also known to constitute a cryptic species complex (Barata et al., 2012; 2015) inhabits the Oukaïmeden Plateau in Morocco. This small lizard was previously used for community studies (Carretero et al., 2006) and different degrees of sexual dimorphism are recognised in different populations. The population of Oukaïmeden, though, is the one presenting less accentuated dimorphism. Expanding the knowledge on the ecology of this species using a dietary assessment would shed a light into niche partitioning and different use of resources (Kartzinel & Pringle, 2015).

To understand food web structures, dietary assessments were initially performed through direct observations of what was eaten in the field, information that is not easy to obtain particularly for generalist predators (Pompanon et al., 2012) or whose prey is difficult to identify visually (Kartzinel & Pringle, 2015). Also, species living in remote places with difficult access are generally challenging to study. Until now, most of the studies relied on morphological identification of consumed prey by either looking at stomach contents obtained through animal sacrifice or stomach flushing (Luiselli et al., 2011), or faecal sample analyses (Perera et al., 2006). However, these methods require considerable taxonomic expertise as prey items are identified through undigested partial remains. Furthermore, softer preys may not be detected due to their high digestibility (Jarman et al., 2013), while prey item identification is frequently limited to the order level (e.g. Perera et al., 2006).

With the recent advent of high-throughput DNA sequencing techniques there is the possibility to obtain vast numbers of sequences in a single assessment. Next generation sequencing (NGS) platforms have the potential to revolutionize scientific knowledge from genomics and transcriptomics including new ways of assessing biodiversity. This approach has been widely applied in the field of microbiology and increasingly used to identify plants, invertebrates and vertebrates from DNA mixtures that are obtained by DNA extraction of heterogeneous samples or environmental DNA as soil or water (Deagle et al., 2014). The amplification of this type of barcodes from DNA mixtures is called 'metabarcoding' (Taberlet et al., 2012; Yu et al., 2012).

Different studies recommend different sets of primers to amplify genetic barcodes and there is an ongoing debate regarding which combinations of barcodes to use (Deagle 2014; Clarke 2014). The mitochondrial protein coding Cytochrome Oxidase I (COI) gene has many characteristics of an ideal marker, since it can be easily amplified from most animals, its high variation often allows species-level identification and the available public databases such as GenBank and BOLD currently present millions of taxonomically verified sequences. On the other hand, it does not contain highly conserved regions suitable for designing truly universal PCR primers (Leray et al., 2013). This fact can lead to the amplification of non-target taxa present in the samples (e.g. bacteria in diet studies) and the non-amplification of preferred taxa (Deagle et al., 2014). Therefore, metabarcoding requires both appropriate taxonomic coverage to minimize PCR bias and sufficient resolution to enable accuracy of species richness inferences (Clarke et al., 2014). Several studies have tried to develop other potential markers than COI to overcome the implications of its high variability. Riaz et al. (2011) developed a software called ecoPrimers to identify non-conventional markers by scanning whole genomes and then finding new barcode markers and their respective primers. This approach has been validated by several authors (e.g., Clarke et al., 2014; Epp et al., 2012; Shehzad et al., 2012) which tested the performance of new primer sets targeting ribosomal RNA genes, 16S rRNA and 12S rRNA. In this respect, the 16S rRNA gene might be a potential solution to increase the coverage of metabarcoding studies, as verified by Clarke et al. (2014) *in silico* and Elbrecht et al. (2016) *in vivo*, when compared with COI markers. In both studies, more insect species were amplified when using primers targeting 16S rRNA regions.

1.2. Metabarcoding

1.2.1. From barcoding to metabarcoding

With millions of species in the world and less and less professional taxonomists, biological specimen identification has become increasingly challenging. Besides, even taxonomic experts may find it difficult to identify a specimen if it is damaged, badly preserved, or still in an immature stage (Pompanon et al., 2012). With genetic barcoding, those problems can be resolved as a small amount of tissue can be enough to identify a specimen to the species level. The mitochondrial Cytochrome Oxidase I (COI) gene has been suggested to have enough resolution to discriminate closely related species of all animal phyla except Cnidaria (Hebert et al., 2003), and therefore a 648 base-pair region of COI is being used as the standard barcode for almost all animal groups due to its high efficiency as an identification system. In plants, however, since COI evolves too slowly, two other gene regions from the chloroplast, rbcL and matK, have been acknowledge as the barcode regions for plants (CBOL Plant Working Group, 2009). Amplifying and sequencing short barcodes is both easy and cheap, providing large amounts of information on species diversity, although Sanger sequencing might be inefficient when it is necessary to amplify thousands of sequences from mixed samples (Yu et al., 2012).

The term “DNA metabarcoding” was introduced in 2012 by Pierre Taberlet and is defined as high-throughput multi-species identification using "environmental" DNA such as that sampled from soil, water, or faeces. Even though this type of strategy had already been used by other authors, it was originally applied to microbial communities studies (e.g. Sogin et al., 2006) while it is now also being used in eukaryote organisms, for example to assess invertebrate diversity (Yu et al., 2012), studying communities of protists (Geisen et al., 2015), and as a palaeoecological tool using soil samples (Epp et al., 2012). The possibility to identify DNA sequences from environmental heterogeneous samples is also shedding light on dietary habits of numerous organisms, such as large herbivores (Kartzinel et al., 2015), carnivore predators (Shehzad et al., 2012) and small mammals (Gillet et al., 2015), fish (Leray et al., 2015; Sousa et al., 2016), bats (Mata et al., 2016), penguins (Jarman et al., 2013) and other bird species (Gerwing et al., 2016), and lizards (Kartzinel & Pringle, 2015). In order to process the massive amount of sequences resulting from this technique, bioinformatic tools are being developed alongside molecular tools in a way that datasets can be manageable with enough quality for downstream analysis (Yu et al., 2012).

Metabarcoding has then a different purpose than barcoding, requiring different criteria concerning the employed markers. Amplicons are preferably shorter, since the DNA will be degraded in most cases, and the primers need to be highly conserved to minimize amplification biases from samples within mixed templates (Riaz et al., 2011).

1.2.2. Which barcode to choose? COI versus 16S

The most critical factor when using DNA metabarcoding for species diversity assessments is to design universal PCR primers able to amplify the target group without amplifying all the other different taxa commonly present in environmental samples (Elbrecht et al., 2016). The choice of marker should be determined by the question addressed and the knowledge about the species being studied (Deagle et al., 2014; Pompanon et al., 2012).

Cytochrome c oxidase subunit I (COI) has been the marker of choice in metabarcoding studies of most animal groups due to its utility in amplifying a vast range of species and the availability of an extensive database covering many taxa. Nevertheless, it has been acknowledged that this protein-coding gene does not contain enough conserved primer binding sites across the tree of life, thus hampering the development of truly universal primers and metabarcoding efforts (Deagle et al., 2014; Leray et al., 2013a). This is because introducing primer biases due to mismatches in the primer binding sites causes the risk of some target taxa to be lost (Elbrecht et al., 2016) and the underestimation of actual biodiversity, at least in some groups such as insects (Clarke et al., 2014). Moreover, due to the codon degeneracy and the occurrence of third codon ‘wobble’ in protein coding genes, some taxa will probably not be amplified when using COI as a metabarcode (Clarke et al., 2014; Elbrecht et al., 2016). For those reasons, it can be problematic to design primers internal to the COI barcoding region, and thus many alternative primer sets have been designed (e.g. Leray et al. 2013). This primer set presents higher degeneracy resulting in a more efficient amplification throughout different taxa. Nevertheless, the non-specific primer annealing could constitute a problem in metabarcoding studies (Leray et al., 2013).

Given the inherent problems of COI marker, other markers have been tested for DNA metabarcoding analysis in animals, such as the more conserved mitochondrial 16S rRNA gene. Clarke et al. (2014) used an *in silico* approach to evaluate the performance of different COI and 16S primers on insect communities (Figure 1). The results showed

that primers targeting 16S rRNA gene were more effective amplifying all tested groups with higher coverage.

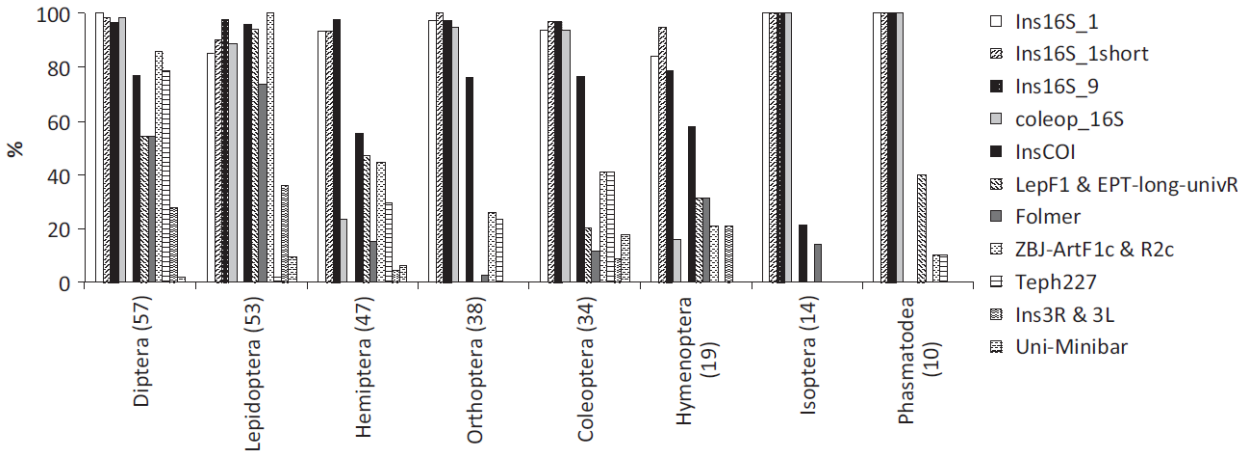

Figure 1 Taxonomic coverage comparison between COI and 16S primers among eight different order. Figure adapted from Clarke et al., 2014.

The main drawback of using 16S as a marker, however, is that the reference databases are lagging behind those of COI hindering taxonomic inferences and conclusions. However, soon this problem is expected to be overcomed by the possibility of generating local 16S reference databases (Elbrecht et al., 2016). Nevertheless, highly degenerated COI primers might be as effective as 16S primers covering a similar number of taxa and at the same time being able to take advantage of the extensive COI available databases (Elbrecht et al., 2016). Essentially, the decision for the most appropriate marker should be based on the goals of the project and the potential tools to work with. If the aim is to obtain a clear taxonomic identification at the species level, COI could be the best option. On the other hand, if species identification is not critical for the study and the main goal is to obtain general knowledge of all present taxa, more conserved markers like 16S rRNA may be more appropriate (Deagle et al., 2014).

The increased advancing of high-throughput sequencing will generate more reference data in the future in the form of complete mitochondrial genomes (Elbrecht et al., 2016) and perhaps databases will be more informative regarding all mitochondrial regions, at least for some groups such as insects. For now, an alternative approach may be to combine a barcode with higher taxonomic coverage but low resolution, with another barcode that identifies higher and unresolved taxonomic units (Pompanon et al., 2012).

1.2.3. Other methodological implications

Besides marker choice, there are other important issues that should be taken into consideration before starting a metabarcoding study.

It is acknowledged that this method has the limitation of only providing a list of the present species and not their relative abundances (Piñol et al., 2014). In many dietary studies, it can be of particular value to know the amount of food from each category eaten by a species. However, the proportion of food items does not correspond to the amount of recovered sequences resulting from NGS techniques due to several biological reasons (Pompanon et al., 2012) including variations in gene copy number, different tissue cell densities (Yu et al., 2012), as well as prey characteristics, such as its digestibility (Jarman et al., 2013) or size. Quantitative data can also be biased by the well-known PCR stochastic effects and primer-template mismatches causing the preferential amplification of certain target DNA, and thereby increasing its relative abundance and the absence of not amplified fragments in the final mixture (Piñol et al., 2014). Faecal and gut samples are also particularly enriched with bacteria and fungus that can be amplified when using universal primers. Besides, PCR errors may lead to taxon misidentification through incorrect nucleotide incorporation and chimeras, and a conservative approach can be taken discarding rare sequences (Pompanon et al., 2012). Finally, technical biases can arise from the DNA extraction, DNA pooling, sequencing and during bioinformatic analyses (reviewed in Pompanon et al., 2012).

Because environmental DNA fragments are usually degraded, length of amplicons that can be effectively amplified is small, which in turn inevitably reduces taxonomic resolution (Pompanon et al., 2012). Therefore, it is very useful to find or build a reference database containing the possible relevant species to be identified with a taxonomic resolution in accordance to the objectives of the study. In this respect, the use of short metabarcodes targeting standardized barcodes could be an advantage over building high-quality reference libraries (Taberlet et al., 2012). One of the steps typically used in metabarcoding data analysis is to cluster the amplicon reads into molecular operational taxonomic units (MOTU) (Floyd et al., 2002). This method allows dealing with both identified (i.e. with a match in a reference database) and unidentified (no match in databases) taxa. Although there is not much investigation regarding the validity of the numerous methods to perform MOTUs clustering (Clare, 2014), it seems that the selected method is extremely important as it will deal with PCR and sequencing errors (Pompanon et al., 2012).

When studying dietary habits, there is the additional problem that prey DNA from faeces or stomach is much more degraded than the predator DNA, and so amplification is likely to be dominated by predator DNA (Vestheim & Jarman, 2008). Sequencing predator PCR products will reduce the sequencing depth of the fragments of interest (Piñol et al., 2014). One way to overcome this problem is by using oligonucleotides that block the amplification of predator DNA (Vestheim & Jarman, 2008). These are specific PCR primers with an extension that binds to predator DNA by preference but is modified with a 3-carbon spacer (C3-spacer) at the 3'-end which will block amplification (Figure 2). If properly designed, blocking oligonucleotides can be very effective and help to amplify prey items that otherwise might be overlooked (Shehzad et al., 2012). However, since these primers will compete with the amplicon specific primers, they should be used at appropriate concentrations in order to be effective. Moreover, they can also introduce some biases in the analysis of dietary samples by potentially blocking prey DNA to some extent (Vestheim & Jarman, 2008).

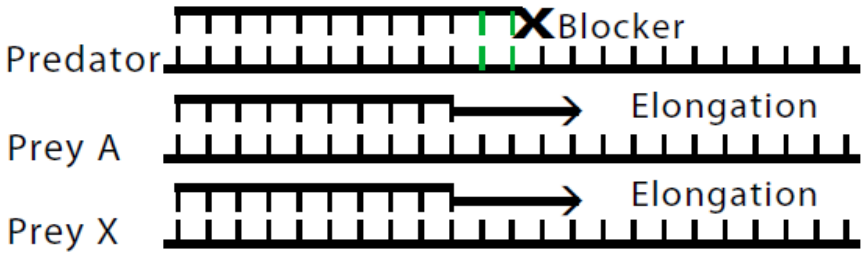

Figure 2 Illustration of the effect of a blocking oligonucleotide in a non-target fragment (predator). Schematic figure from Vestheim & Jarman, 2008.

1.3. Objectives

Insights into niche partitioning can reveal the origin of intraspecific competition in certain lizard populations (Kartzinel & Pringle, 2015) and the use of high throughput sequencing might be helpful in accurately identifying insect taxa reducing the time and costs involved (Clarke et al., 2014). Our aim with this study is to use a metabarcoding approach to investigate diet partitioning in lizard. Previous studies have shown that species endemic to the Atlas Mountains in Morocco may be species complexes, as is the case of the lizards *Scelarcis perspicillata* (Perera et al., 2007) and *Atlantolacerta andreanskyi* (Barata et al., 2012). Despite the knowledge that *S. perspicillata* encompass

genetically divergent phenotypes which can live in sympatry, little is known regarding the ecology of the phenotypes. Specifically, our research will focus on two case studies: 1) diet partitioning between two syntopic subspecies of *Scelarcis perspicillata* – *S. p. pellegrini* and *S. p. chabanaudi* (Manuscript I), and 2) ontogenetic and sex differences in the diet of *A. andreanskyi* (Manuscript II).

2. Material and Methods

2.1. Sampling

In September 2016, a field trip to Morocco was conducted covering the study areas of Oukaïmeden and Taza. Oukaïmeden Plateau (31°12'32"N, 7°52'52"W) is located in the High Atlas Mountains at 2600 m.a.s.l. and its habitat is characterized by the presence of a mountain lake surrounded by stone walls and meadows which are used for grazing (Figure 3a). A total of 55 specimens of *A. andreanskyi* were captured by hand in Oukaïmeden in a rainy day, and thus they were mostly found under rocks since the weather conditions were not favorable for reptile activity. Taza is located in north-central Morocco and the animals were collected specifically in the Tazekka National Park (34º12'94''N, 4º3'14''W), which is an extensive area dominated by trees, shrubs, and big rock blocks (Figure 3b). Twenty-five individuals of *S. p. chabanaudi*, 28 of *S. p. pellegrini,* and 15 of *Podarcis vaucheri* were captured by noosing throughout the sampling site, where they were found active in the trees, inside rock crevices and holes, and on the ground (list of samples detailed in Table S2, Supplementary Material).

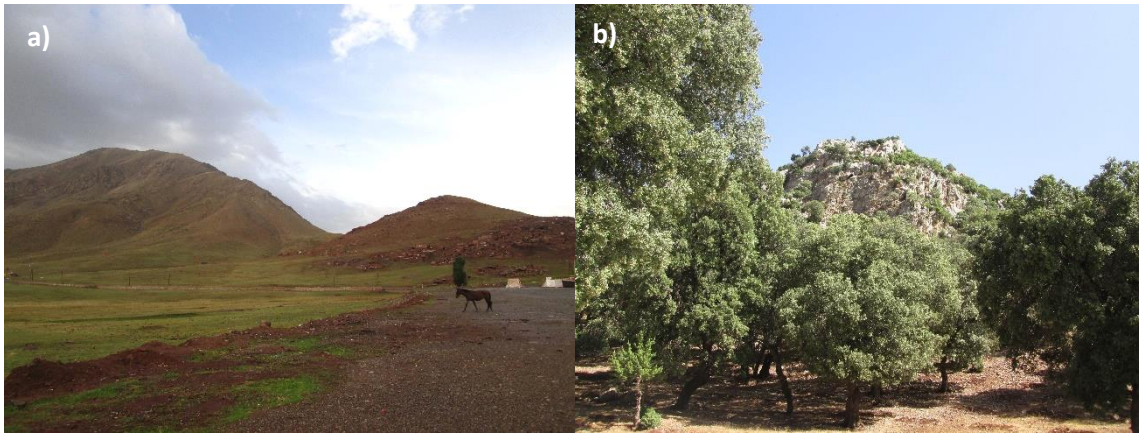

Figure 3 Two pictures representing the distinct habitats of the study areas; a) the Oukaïmeden Plateau, and b) Tazekka National Park (Taza).

In both localities, all animals were identified to species level in the field and GPS coordinates of the capture site were taken. If lizards defecated immediately after capture, faeces were collected directly into tubes with 96% ethanol and assigned to the respective specimen. If not, lizards were kept in individual bags for some hours (3/4) until they defecated. Sex was determined for all specimens by the presence of developed femoral pores and head robustness in males (Perera et al., 2006; 2007; Barata et al., 2015). The

two forms of *S. perspicillata* were unequivocally distinguished by the spotted pigmentation of *S. p. chabanaudi* and the stripes of *S. p. pellegrini* (Perera et al., 2007). Although *P. vaucheri* could be potentially confounded with *S. p. pellegrini*, the latter presents ten ventral scale rows, while *P. vaucheri* has only six (Schleich et al., 1996). To avoid biases, animals were measured (snout-vent length) to the nearest millimeter and photographed by the same person. The tip of the tail was collected and stored for future DNA extraction. All lizards were released unharmed at the place they were found. The samples were preserved at ambient temperature until the arrival to the lab.

2.2. Primers choice and blocking oligonucleotide design

A preliminary study was performed testing primer sets used in other studies (Table S1, Supplementary Material) in order to find the most suitable primer pairs for this study.

Although the use of a potential prey mock community to create a reference database from the studied ecosystems appeared to be the most recommended procedure for metabarcoding dietary analysis (Kozich et al., 2013), we did not have the chance to build one due to the limited time in the field. To overcome this limitation, the most consumed preys reported from previous studies (Carretero et al., 2006; Perera et al., 2006) and representing 5 invertebrate orders (Coleoptera, Orthoptera, Diptera, Odonata and Hemiptera) as well as other potentially consumed prey as Scorpiones were amplified to test the efficacy of the selected primer pairs. Additionally, lizard faecal samples from previous fieldtrips to Morocco were amplified. Host DNA from *S. p. chabanaudi*, *S. p. pellegrini*, *P. vaucheri*, and *A. andreanskyi* were also included in reactions for a preliminary evaluation of host co-amplification.

Based on the results of these tests, blocking primers were designed to prevent host amplification. These primers were designed to overlap with the 3' end of the universal COI and 16S primers and extended into the species specific sequence with a C3' spacer modification at the 3' end to stop host amplification, following Vestheim and Jarman (2008). Since blocking primers can also block prey DNA amplification to some degree, those were aligned with sequences of possible preys to ensure the maximum number of mismatches between them when designing the primer. One single blocking primer was used for the amplification of the 16S fragment for the four study species due to their low variability in that region, whereas for the COI fragment amplification three different blocking primer pairs were designed (one for each species with the exception

of *P. vaucheri*). We did not use any host DNA removal strategy for the faeces of *P. vaucheri* due to lack of knowledge on the overlap region in the 3' end of the COI fragment and to time constraints.

Melting temperatures (Tm) were calculated for each blocking primer using Oligo Calc (http://biotools.nubic.northwestern.edu) to select the annealing temperature for the amplification. Different blocking primer concentrations were tested (10x, 15x and 20x) relative to universal primers. Results were compared to a similar PCR of prey and host with no blocking primer to test the effectiveness of the reaction. The best concentration was chosen when host amplification was reduced without the loss of amplified products of the target invertebrate samples.

2.3. DNA Extraction

DNA extraction was performed in the non-invasive samples extraction room at CIBIO's laboratories to minimize cross-contaminations. This is a positive controlled pressure room designed to limit contaminations from the outside environment.

Prior to DNA extraction, samples were dehydrated at 37ºC overnight and were extracted in groups of the same species to minimize contamination interspecies. When more than one pellet per individual was available, they were pooled to increase the probability to detect the highest number of prey (Pompanon et al., 2012). DNA was extracted using the PureLink Genomic DNA kit (Thermo Fisher Scientific), which is spin column-based, following the manufacturer's protocol with some modifications in the digestion (first) step. Those included the triplication of lysis buffer and pK quantities to improve DNA extraction from all type of tissues (hard and soft). All samples were vortexed to disrupt the faecal mass and digestion occurred overnight.

Extracted DNA was eluted with 200 µL of elution buffer and aliquoted. Ten 10 µL of each sample were stored separately for tests and preliminary analyses and the rest of the DNA remained frozen at -20ºC until the library preparation.

2.4. Library preparation and sequencing

A two-step PCR approach was performed to first amplify the target regions of 16S rRNA and COI and then attach the specific tag to each individual (Figure 4) that allows all the samples to be pooled before sequencing (following the protocol of Kozich et al.,

2013). The primer pair Ins16S_1F and Ins16S_1R (Clarke et al., 2014) was used to amplify the 16S rRNA fragment and 15x of blocking primer relative to the 16S primers

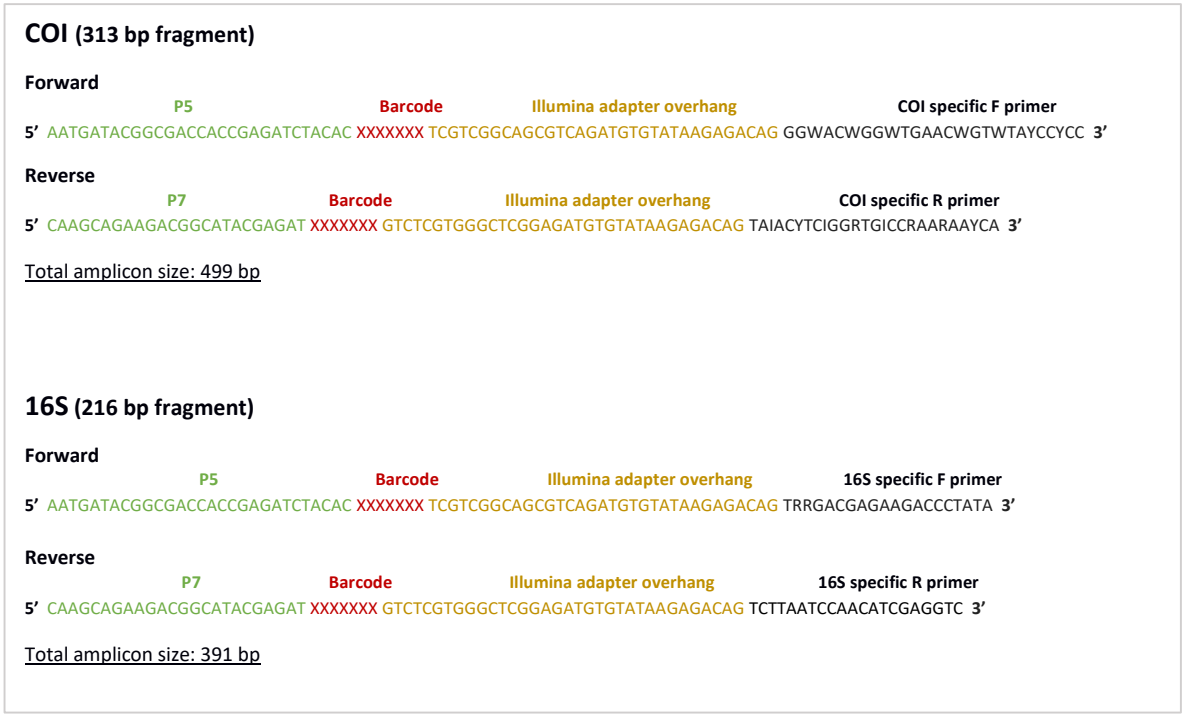

Figure 4 Schematic figure of the dual indexing strategy used in this study for both amplicons (COI and 16S rRNA).

was the most efficient concentration to prevent host amplification. Due to the high blocking primer melting temperature, a touchdown PCR was done starting with 65ºC of annealing temperature and decreasing 0.5ºC per cycle until it reached 55ºC, where it ran for 25 cycles. A versatile primer set was chosen to amplify the COI fragment, mlCOIintF and jgHCO2198 (Leray et al., 2013), combined with 20x blocking primer concentration. A touchdown PCR was also performed with the annealing temperature decreasing 1ºC each cycle from 67ºC to 46ºC and then amplifying 25 cycles.

All the PCR reactions contained Bovine Serum Albumin (BSA) 25 mM and the concentration of $MgCl_2$ was optimized to 2.5 mM since it amplified more samples due to the diminishing of the reaction specificity. Platinum Taq Polymerase (Invitrogen) (5U/µl) was mixed in a 10 µL reaction volume with 0.5 µL of DNA. Samples were run in triplicates in an Applied Biosystems Veriti Thermal Cycler (Thermo Fisher Scientific) with a negative control (blank), which contained all the reagents except DNA, a positive control (a prey sample), and a host sample to check the effectiveness of the blocking primer. Amplified fragments were mixed with bromophenol blue, tested in 2% agarose gel with GelRed Nucleic Acid stain and visualized in an ultraviolet transilluminator. Replicates for each

sample were pooled, except for the replicates that failed to amplify, which were not included.

The following step was PCR clean up using the Agencourt AMPure XP (Beckman Coulter) purification system with a proportion of 0.8 µL of magnetic beads to 1 µL of PCR product. This ratio allowed the removal of primer-dimer and unincorporated reagents in the reaction. Purified DNA was resuspended in 10 mM Tris pH 8.5 and diluted 1:3 in ultrapure water.

To attach the barcodes in each sample, a second PCR was performed with a unique combination of barcodes per sample. Platinum Taq Polymerase (5U/µl) was used and the cleaned amplicons were amplified for 10 cycles with an annealing temperature of 55ºC. In order to confirm the success of the barcodes incorporation in all samples, they were tested in a 2% agarose gel with the *a priori* knowledge that amplicons should be ~100 bp longer than before this second PCR. Before normalization, a final purification was performed using the ratio of 1.2 µL of beads to 1 µL of PCR. Quant-iT™ PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific) was used to quantify amplicon concentration and all samples were normalized to 15 nM and then, finally pooled with 2 µL of each sample.

The pool was sent to GENEWIZ Next Generation Sequencing laboratory to be sequenced in an Illumina MiSeq sequencer with 2x250bp paired-end (PE) configuration. In the GENEWIZ lab, sample quality control was performed using Qubit dsDNA Assay and PhiX (≤30%) was spiked-in to increase sequencing diversity. A final qPCR was performed to confirm the actual concentration of the pool.

2.5. Bioinformatic analysis

Once results were available, samples were de-multiplexed and adaptors were removed as part of the service provided by GENEWIZ. From this step on, COI and 16S samples were processed independently. Fastq files were analyzed using USEARCH v9.2.64 (Edgar, 2010).

The overall sequencing quality was checked and number of reads, expected error (EE) distribution and length distribution were summarized. Reads R1 and R2 were separately reviewed since forward and reverse reads might have different qualities. After quality control, primers were removed. Paired-end reads were assembled using the command *-fastq_mergepairs* and then filtered by quality. For that, the command

-*fastq_filter* was used and sequences with less than 100 bp were discarded (Yu et al., 2012) in the same step. Next, reads were filtered by quality scores where maximum expected sequencing errors was set to 1 (Edgar & Flyvbjerg, 2014) based on the overall good sequencing quality. Then, in order to find the set of unique sequences, -*fastx_uniques* was used and singletons were removed. With the command -*cluster_otus*, unique sequences were clustered into Operational Taxonomic Units (OTUs) using the UPARSE algorithm (Edgar, 2013). This method relies on a greedy algorithm to find the representative set of OTUs from the given input wherein centroid sequences are selected from the most abundant reads. In this step, chimeric sequences were discarded and all the sequences should match with at least one OTU with ≥ 97% of identity. Lastly, -*usearch_global* was used in order to create an OTU table with the frequency of all OTUs for each sample.

Next, sequences from 16S rRNA were compared against the NCBI database using the implemented BLAST algorithm, and sequences from COI were compared with both NCBI and BOLD databases. When a match up to the order level was not found, OTUs were considered unidentified. For each sample, OTUs representing less than 0.5% of the total number of reads of that sample were removed (following e.g. Kelly et al., 2014). Once OTUs were taxonomically classified and the data cleaned, proportions of each food taxa were calculated for each species and age/sex, according to the case study.

3. Manuscripts

3.1. Manuscript I

DNA metabarcoding to assess diet partitioning of three syntopic lacertid lizards from Morocco

Abstract

Trophic niche interactions are fundamental to understand community structures and diet studies can be highly informative regarding niche use by different species. Two lacertids endemic to Morocco live in strict syntopy in Taza: *Scelarcis perspicillata chabanaudi* and *S. p. pellegrini,* while maintaining morphological and genetic integrity. In order to avoid competition, these forms are expected to segregate their niches adopting different foraging strategies. Previous studies have found differences in diet composition of the two forms when living in syntopy, which we hypothesize to be a result of their different dorsal pigmentations. The form *S. p. chabanaudi* presents a cryptic spotted dorsal pattern and is therefore expected to be a sit and wait forager with a less diverse diet and higher prevalence of mobile prey items. On the other hand, *S. p. pellegrini* due to its striped pattern was considered to adopt an active foraging strategy, having a higher diet diversity and feeding upon more sedentary and clumped prey. In Taza, a third lacertid, *P. vaucheri*, is living in syntopy with *S. perspicillata*. We expected this species to be an active forager due to its similarity with *S. p. perspicillata* in the dorsal pattern and we aim to inspect to which extent their niches overlap. Because previous studies were performed in 2003 and 2004 using traditional microscopic approaches, in the present study we aim to confirm whether diet composition remained similar. Moreover, since we expect to have higher taxonomic resolution we will be able to determine whether the diet reflects different foraging strategies inferred from differences in pigmentation pattern. For that, a total of 68 faecal samples were collected and a metabarcoding approach was performed using COI and 16S rRNA markers. The most common prey found among the three species was Coleoptera (present in 56% of the samples), and six more orders could be identified (Blattodea, Diptera, Hemiptera, Hymenoptera, Lepidoptera and Orthoptera). As expected, the diet of *S. p. chabanaudi* was less diverse than the one from *S. p. pellegrini*, especially when comparing consumed prey identified to the family level. However, the detection of clumped prey as

Curculioninidae and Kalotermitidae for *S. p. chabanaudi* plus the presence of flying prey items in the diet of *S. p. pellegrini*, led to the conclusion that they are not strictly sit and wait or active foragers. Regarding *P.* vaucheri, our preliminary data suggests it might behave as a sit and wait forager due to the higher similarity of its diet with *S. p. chabanaudi*. Metabarcoding has shown to be an effective method to study the diet of these lizards, although some limitations such as the distinction between different life stages need to be overcome.

Introduction

Dietary assessments are essential when studying reptiles' ecology to fully understand fundamental aspects of trophic niche utilization or feeding behaviours (Vanhooydonck et al., 2007). Optimal foraging theory suggests individuals are expected to select food items based on their profitability in order to obtain the maximum energy needed at the lowest cost possible (MacArthur & Pianka, 1966). When profitable and abundant prey items are present, predators are expected to select them instead of less profitable food they might encounter instead, as predicted by the Optimal Diet Model (Stephens et al., 2007). The selection of such profitable preys is of extreme importance for growth, fitness and reproduction, which affect other important species traits such as body size (Costa et al., 2008). Even though most lizards are considered generalists (Díaz & Carrascal, 1995), feeding habits are expected to vary between species and depend on the adopted foraging strategy as well as on prey availability, mobility, behaviour, size or hardness, among others (Vanhooydonck et al., 2007).

The Moroccan rock lizard, *Scelarcis perspicillata* (Duméril & Bibron 1839), is a small endemic lacertid inhabiting the mountain areas of Morocco and western Algeria (Bons & Geniez, 1996; Schleich et al., 1996), with an introduced population in Menorca, Balearic Islands (Perera et al., 2002). *Scelarcis perspicillata* is a very agile lizard and an excellent climber, living in walls, fissured rocks, cliffs, and trees (Schleich et al., 1996). This species is primarily insectivorous and feeds mainly on beetles, ants, spiders, flies, small moths, but also, sporadically, it feeds on fruits upon availability (Perera et al., 2006; Schleich et al., 1996). Bons (1968) recognized three subspecies, *S. p. perspicillata*, *S. p. pellegrini* and *S. p. chabanaudi*, which correspond to three distinctive morphs with different size, colour pattern and distribution. This classification is, however, still being debated since phylogenetic analysis reveal a complex relationship among these forms, with a lack of congruence between the three colour morphs and the phylogenetic

patterns (Harris et al., 2003; Perera et al., 2007). Two of these forms, *S. p. chabanaudi* and *S. p. pellegrini* (hereafter called *chabanaudi* and *pellegrini*), are known to live in strict syntopy in Taza, Morocco, and to belong to two genetically distinct clades (Harris et al., 2003). *Chabanaudi* is the largest form with an average snout-vent length (SVL) of 61 mm and is characterized by having light spots on a black background, while *pellegrini*, with an average SVL of 54 mm, presents two light dorsolateral stripes on a spotted dorsum (Schleich et al., 1996; Bons & Geniez, 1996) (Figure 1). So far, after 14 years of sampling, no individuals with an intermediate morphology have been found at this locality and there is a full match between external colour pattern and genetic identification (Perera et al., 2007), suggesting that gene flow between the two morphs, if any, is limited, which in turn indicates they behave as two distinct species (Carretero et al., 2006; Perera et al., 2007). Interestingly, a previous study on *S. perspicillata* diet showed higher differences between the diet of *chabanaudi* and *pellegrini* when living in syntopy than in allopatry, which might be explained by the use of different foraging strategies to reduce competition (Perera et al., 2006). This work was focused in the microscopic analysis of faecal samples collected in the spring of 2003 and 2004.

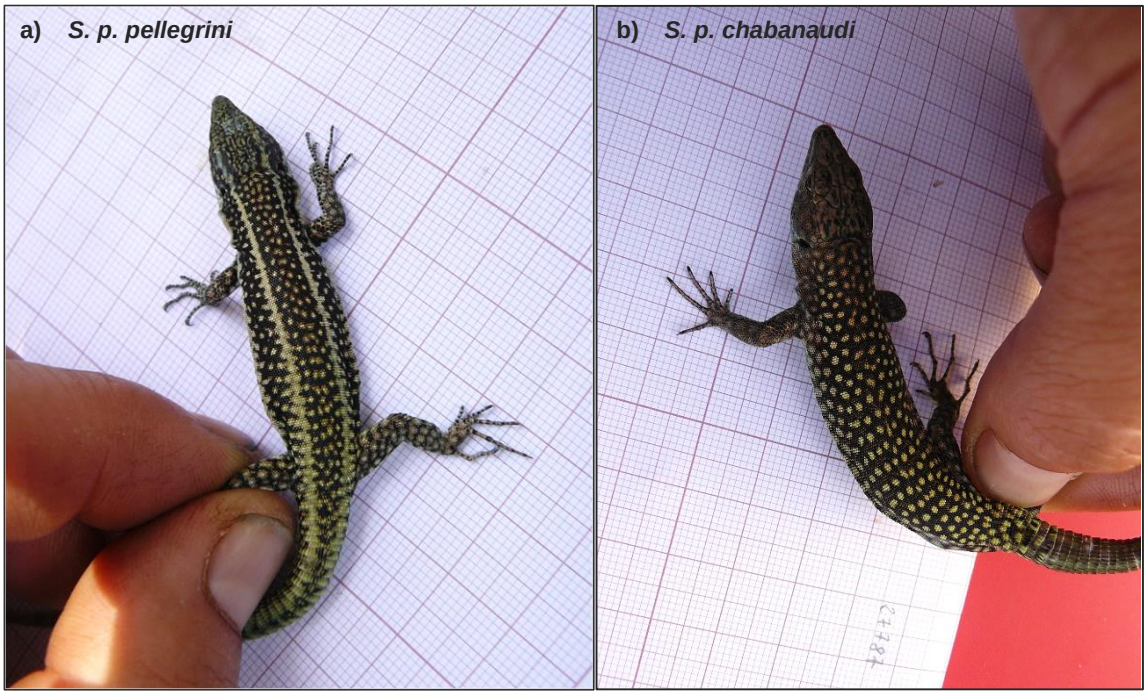

Figure 1 Different pigmentation patterns of the two forms of *S. perspicillata* present in Taza, in which a) represents *pellegrini* and b) *chabanaudi*. Picture taken by Daniele Salvi.

Although some authors recognize a continuum instead of a dichotomous view of foraging modes (e.g. Perry, 1999), there are two basic widely accepted foraging modes originally described by Pianka (1966): sit and wait (or ambush foraging), and widely foraging (also called active foraging). Sit and wait predators have a sedentary foraging

strategy, in which they wait for prey to approach to attack them; active foragers, on the other hand, are in constant movement looking actively for prey (Huey & Pianka, 1981). Since they move throughout extensive areas, active foraging lizards are more likely to feed on sedentary, clumped insects like larvae and termites (Pianka, 1966) as well as finding otherwise inaccessible and unpredictably distributed prey. Conversely, sit and wait foragers are expected to eat more active prey (Huey & Pianka, 1981; Verwaijen et al., 2007). These differences in encountered prey are often a reflection of the morphology of the lizards since sit and wait lizards may depend upon higher acceleration capacities to capture moving prey (Cooper, 2007) and broader, longer heads for increased bite forces along with reduced time processing the prey (Verwaijen et al., 2007). On the other hand, active foragers meet smaller, softer prey which do not require such strong bite force and larger heads (Huey & Pianka, 1981; Pianka, 1966), and are also expected to be faster due to their longer exposure time to predators during foraging (Verwaijen et al., 2007). Moreover, since widely foraging predators are actively looking for prey, they are expected to find a higher diversity of prey than sit and wait foragers. Lizards spend most of their time foraging, adopting antipredatory strategies determined by the type of their potential predators (Huey & Pianka, 1981). Indeed, Carretero et al. (2006) found different predator escape tactics between *chabanaudi* and *pellegrini* when living in syntopy in Taza. In this study, under a simulated predator attack, *chabanaudi* seemed to choose bigger rocks, which make them more susceptible to bird attacks but less accessible to terrestrial predators, while *pellegrini* would select more fragmented rocks facing more terrestrial predation. Lacertid lizards are mainly active foragers (Cooper & Whiting, 1999), although some authors have found both active and sit and wait foraging strategies in lizards from the same genus (Huey & Pianka 1981; Perry, 1990). This suggests that both foraging modes may also be found in individuals from the same species or closely related competing for resources in the same locality.

Dorsal pigmentation patterns are known to play an important role in escaping predators, especially when associated with other movement behaviour (Jackson et al., 1976). Cryptic body coloration such as reticulated, spotted, or uniform patterns, have the advantage of reducing the probability of detection by predators, since they blend with the natural background, working as a camouflage mechanism (Halperin et al., 2017). Conversely, conspicuous pigmentation, such as stripes, that might seem disadvantageous in most environments, can perform well during movement (Stevens et al., 2011) since longitudinal stripes and high contrast patterns interfere with the predator's perception of speed and trajectory of the prey when its moving, creating a 'motion dazzle' phenomenon (Jackson et al., 1976). Consequently, species with striped

patterns are expected to be more mobile and able to confuse predators during an attack, thus increasing their chances to survive (Halperin et al., 2017). On the other hand, species with cryptic coloration patterns should present lower mobility in order to reduce the probability of being detected (Halperin et al., 2017). Lizards with intermediate patterns are thought to select one of these extreme strategies instead of presenting an intermediate behaviour (Hawlena et al., 2016). There is an associated evolution of foraging behaviour and morphology that define lizards' detectability to predators and thus, active foraging modes are usually associated with striped dorsal patterns whereas sit and wait predators should have cryptic pigmentations (Halperin et al., 2017).

In fact, integrative studies have shown that species specific traits are intimately related with foraging modes (e.g. Cooper, 2007; Costa et al., 2008) and so, morphological, physiological and behavioural characteristics can be more easily interpreted if foraging modes are known. Conversely, foraging behaviours can be generalized when life history traits, such as diet, are measured (reviewed in McLaughlin, 1989). Initially, dietary studies relied on direct observations of feeding habits and microscopic analysis of gut contents or faeces. Besides requiring considerable taxonomic expertise, these methodologies are time consuming and might be inaccurate when the consumed prey belong to taxonomically complex groups or are highly digestible (Pompanon et al., 2012; Taberlet et al., 2012). With the advance of barcode molecular techniques which can be used for species identification, fast and simple alternatives to traditional methods became available to detect and identify most part of the existent DNA in digested samples. DNA metabarcoding has been shown to be effective in recovering previously undetectable items and thus, complementing already existing information acquired with conventional methods (e.g. Kartzinel et al., 2015; Jarman et al., 2013; Shehzad et al., 2012). Although the use of faecal samples for diet studies might seem unreliable due to extensive digestion processes, some authors still consider it trustworthy since it provides similar information as stomach contents with the additional advantage of being non-invasive (e.g. Pérez-Mellado, 2011). The analysis of diet based on faeces using DNA metabarcoding approaches should represent an improvement to the traditional microscopic examination, as even small portions of the DNA of digested prey can be detected and amplified. Nevertheless, metabarcoding experiments need to be properly designed since several biases can arise from this approach depending on PCR amplification (Piñol et al., 2014), and various aspects need to be considered, such as the choice of the barcode to use. The cytochrome oxidase subunit I (COI) gene is the marker frequently chosen for animal barcoding due to the availability of extensive taxonomically verified databases, easy amplification in almost all

taxa and higher substitution rate compared to nuclear rRNA genes, which should increase its resolution (Yu et al., 2012). The high mutational rate of protein coding genes can, however, constitute a problem regarding primer design, especially when working with genetically diverse groups, such as insects (Clarke et al., 2014). In fact, it can be challenging to find conserved primer binding sites within the 658 bp COI barcoding fragment, although some options combining highly degenerate primers with the standard classical Folmer (1994) primers are available (e.g. Leray et al., 2013). Other markers such as 16S rRNA have also been tested (Clarke et al., 2014), showing a higher taxonomic coverage with the detection of more taxa (Elbrecht et al., 2016). Nevertheless, reference databases for this marker are still limited. For this reason, the use of local reference communities to create a reference database, where a defined combination of relevant taxa samples is artificially mixed, has been proposed (Elbrecht et al., 2016).

The main goal of the present study is to assess differences in foraging tactics between the two syntopic and closely related forms *pellegrini* and *chabanaudi* using a metabarcoding approach to evaluate dietary differences. Due to the striped body pattern of *pellegrini* and the conspicuous pigmentation of *chabanaudi* (Perera et al., 2007) together with their different escape tactics (Carretero et al., 2006), we predict *chabanaudi* to be a sit and wait forager and *pellegrini* an active searcher. These traits are thought to influence the consumption of different prey items. In addition, we have investigated the diet composition of *Podarcis vaucheri* (Boulenger, 1905) which is also present at the same locality. *Podarcis vaucheri* is a small lacertid lizard widely distributed throughout the Mediterranean part of the Maghreb, southern France and Southern Iberia (Bons & Geniez, 1996; Schleich et al., 1996). Its basic coloration is constituted by a green, yellow or brown ground colour with a pattern of light and dark lines being potentially confounded with *pellegrini* due to their similar body size, pigmentation pattern and because they both may live on rocks (Schleich et al., 1996). For this reason, we expect *P. vaucheri* to have a more similar diet composition with *pellegrini* than with *chabanaudi*.

Material and Methods

Sampling details

A total of 68 specimens were included in the study, from which 25 individuals were *chabanaudi*, 28 *pellegrini* and 15 *P. vaucheri*. All of them were captured in Taza, Morocco, in September 2016. The specimens were found active in trees, shrubs, rock crevices and on the ground, and captured with a noose. Lizards were identified, faecal

pellets were collected, standard measurements taken (see previous chapter) and the specimens were then released unharmed at the point of capture.

Metabarcoding Methods

(Detailed in chapter 2 - Material and Methods)

Results

Sequences analysis

From the 68 samples collected, 50% of the samples (n=34) were successfully amplified with the 16S primers and almost 70% (n=47) were successfully amplified with the COI primers (details in Table 1). Assembling forward and reverse reads produced 575,679 sequences for 16S and 752,611 for COI, of which after quality filtering, 563,974 and 688,538 respectively, were recovered. Of these, 44,567 unique sequences were obtained for 16S and 97,981 for COI.

Table 1 Number of individuals from each species captured and successfully amplified for 16S and COI genes.

	Captured	Amplified	
		16S	COI
S. p. chabanaudi	25	12	14
S. p. pellegrini	28	15	20
P. vaucheri	15	7	13

After removing non-food items (lizard, plants, bacteria, fungi and other contaminants), 45% of the samples amplified with COI primers ended up with less than 100 reads per sample due to preferential amplification of non-dietary items. Therefore, since the dataset was not representative enough for each species, the sequences resulting from COI amplification were not included in the downstream analysis.

In the analysis of 16S sequences, one sample of *pellegrini* was removed after quality filtering. Moreover, another sample from *chabanaudi* constituted exclusively by *Androctonus gonneti* DNA was also removed due to a strong suspicion that it was a lab contamination, since this species is exclusive of desert areas from Africa, and the

haplotype was identical to one previously amplified at the same laboratory (Lourenço et al., 2009; Coelho et al., 2014). Sequences clustering resulted in a total of 84 identified OTUs, from which 18% belonged to host sequences, protists, and sequences with low similarity with reference databases and which were therefore, impossible to assign to any group at the order resolution. After the removal of OTUs representing less than 0.5% of each sample (which were excluded as potential contaminants), a total of 45 OTUs identified to the order level were recovered. Of those, 33 could be assigned to the family level, 13 to genus and five to species level.

Diet composition

Globally, the most common prey among all the studied species was Coleoptera (present in 19 individuals, 56%), followed by Hymenoptera (47%). Within the Hymenoptera order, Formicidae, which was identified separately from the other families of Hymenoptera given their clumped habits, was present in 44% of the samples. The other consumed prey, although less frequent, belong to the orders Blattodea, Diptera, Hemiptera, Lepidoptera and Orthoptera. Detailed number of occurrence for all orders across all each species is represented in Figure 2. Interestingly, COI sequences revealed the additional presence of Araneae, although its occurrence was not calculated like the other prey items for the reasons mentioned above. Following a conservative approach, we did not consider items belonging to Sarcoptiformes order (mites) as prey items, which were amplified for several samples, as they can be a result of secondary consumption.

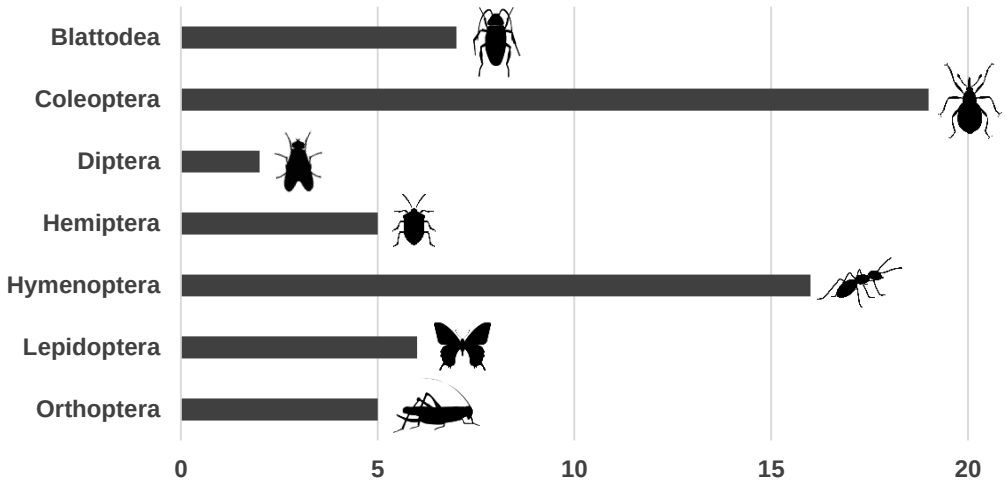

Figure 2 Number of occurrence of prey items among all samples classified to the order level and amplified with 16S primers.

Moreover, sequences classified as molluscs were categorized as non-dietary items since we were not able to identify them to a higher taxonomic classification.

Considering OTUs identified to the order level, all prey items had similar occurrence among the three species, with the exception of Diptera, which was only present in *pellegrini*. Identification to the family level showed that *pellegrini* is the species with the most diverse diet with the identification of 12 prey families whereas *chabanaudi* and *P. vaucheri* only consumed items from 6 different families (Figure 3). The diet of *chabanaudi* lizards was mainly composed by beetles from the Curculionidae family (weevils) with an occurrence of 62%, and Kalotermitidae (termites) with 31% of occurrence (Figure 3). Regarding the diet of *pellegrini*, Formicidae (56%) and Curculionidae (38%) were the most consumed prey.

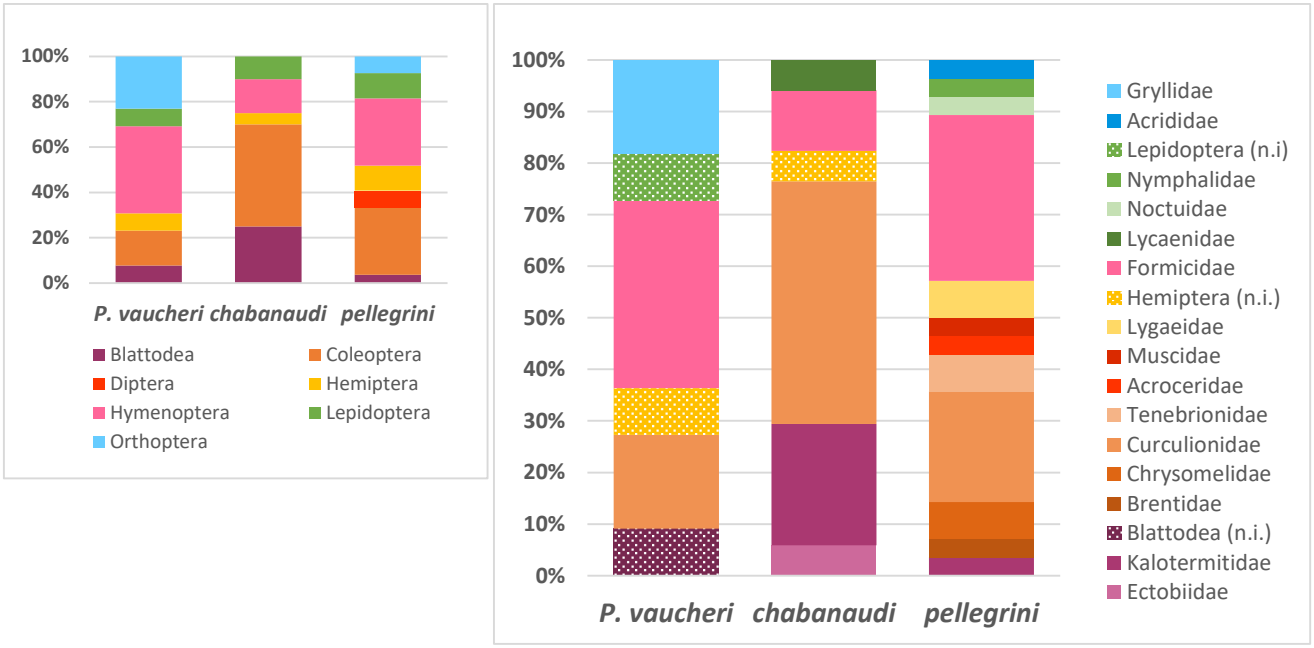

Figure 3 Frequency of occurrence of prey items found in *P. vaucheri*, *chabanaudi* and *pellegrini*. Left side graphic represents the identified orders, and on the right side families are represented. Families from the same order are represented with same colours used on the left side graphic.

Representatives of the Chrysomelidae family (beetles) were also present in two *pellegrini* samples. This family includes small to medium sized species commonly known as leaf beetles, one of which could be identified as the genus *Longitarsus sp* (flea beetle). These are very small and jumping beetles which can even fly, confirming the overall preference of *pellegrini* for small prey. Ectobiidae (cockroaches) are comparably bigger preys and were only consumed by *chabanaudi,* as well as Gryllidae (crickets) that was only consumed by *P. vaucheri*.

Discussion

The results from this study are in accordance with the first studies on *S. perspicillata* diet, showing that this species is mainly insectivorous (Schleich et al., 1966, Perera et al., 2006), and that the most consumed prey are Coleoptera and Hymenoptera. Even though our results cannot be compared in a straightforward manner to the ones obtained by Perera et al. (2006) due to differences in sampling season (September 2016 vs April 2003, 2004), both studies confirm the existence of differences between the diet of *chabanaudi* and *pellegrini*.

Unlike the work of Perera et al. (2006), in this study we could identify the majority of OTUs to the family level. Our results indicate that the diet of *chabanaudi* was mainly composed of clumped prey, such as Curculionidae and Kalotermitidae. According to theory, active foragers predate more frequently on aggregated and patchy distributed prey (Huey & Pianka, 1981). The frequent consumption of termites and weevils by *chabanaudi* seems to suggest that *chabanaudi* also behaves as an active forager, contrarily to our expectations. However, it is noteworthy that some studies reported consumption of clumped prey by sit and wait foragers (Cooper et al., 2000). The consumption of termites by the three species, and specially its higher occurrence in *chabanaudi*, also highlights the fact that the studied lizards are opportunistic feeders since termites are considered poor resources given their low energy density and for the reason that they are difficult to digest as a result of their high chitin content (Cooper & Whiters, 2004). Whether the high intake of termites and weevils reflects their high abundance in Taza or they are actually actively selected by these lizards remains uncertain without prey availability studies. Although Perera et al. (2006) did not detect the presence of termites, this difference might be due to their high digestibility and thus, by a bias in the detection under the microscope.

The diet of *pellegrini* was composed of four flying prey families, whereas *chabanaudi* only consumed one. Since consumption of flying prey is associated with sit and wait foraging strategies (Huey & Pianka, 1981), our results suggest that *pellegrini* is not strictly an active forager as we had predicted. However, like most part of the insects, flying prey from the orders Lepidoptera, Hymenoptera and Diptera undergo a process of complete metamorphosis, and using a metabarcoding technique we are not able to distinguish among different life stages. Therefore, it is possible that flying prey were actually consumed in earlier non-flying stages rather than as adults. However, in some of the pellets used for DNA extraction we observed wings, confirming at least in some

cases, consumption of adult forms. Variations in food availability or predation pressure can make lizards change their foraging modes. A good example of this is the behavioural change of the Kalahari gecko which is normally a sit and wait forager, but switches to actively foraging when termites swarm (Huey & Pianka, 1981). Another example is that of the actively foraging lizard *Acanthodactylus beershebensis* that behaves more like a sit and wait forager when predation pressure increases (Hawlena & Perez-Mellado, 2009). Changes in land use and landscape have also been demonstrated to influence a switch in foraging modes by the Aegean wall lizard, which changes to be more sit and wait forager in human-built rock walls (Donihue, 2016).

Besides differences in sampling year and season, several other reasons might be behind the differences observed with the previous study (Perera et al., 2006), where a higher variety of prey orders was reported (seven in this study vs eleven in the previous study). One of them is the potential preferential amplification of specific taxa, i.e. PCR biases (Deagle et al., 2014). However, such biases are expected to be overcome in the future by the increased sequencing depth of recent technologies. The other technical reason for differences in the results is that rare prey items can be undoubtedly considered as food when using microscopy, whereas in the case of high throughput sequencing, those sequences can be mistakenly removed from the dataset during filtering steps. Indeed, the detection of a lower number of arthropod orders in this study compared to Perera et al. (2006) can be a result of the data processing, at least in punctual cases. However, it is more likely that changes in resources availability, related to interannual and seasonal differences, or changes in predation pressure, land use or competition might be behind the broad differences found between the two studies. In fact, several visits to the study area by members of the team over the last 15 years confirmed big changes in terms of land use in that locality. Moreover, changes in the distribution of the species were also observed, which might imply changes in interspecific competition and interaction with different predators. The possibility of classifying a large percentage of the consumed prey to the family level allowed the detection of fundamental differences among the studied species. The fine resolution to the family level provided by metabarcoding is of major importance since orders such as Coleoptera contain over 200 families with thousands of genera and species, and being able to identify prey with more resolution within the order can help to better infer feeding strategies of their predators. For instance, Chrysomelidae are in general small beetles and the consumption of prey from this family exclusively by *pellegrini* might indicate a preference for small prey items, and reflects its ability to capture flying prey. However, conclusions based on prey size cannot usually be taken when using metabarcoding, although Perera

et al. (2006) did not find significantly differences in the mean size of consumed prey. The high prey diversity found for *pellegrini* compared to *chabanaudi* supports our hypothesis that at least in sympatry, *chabanaudi* adopts a sit and wait foraging strategy although it also predates opportunistically given the high frequency of termite consumption; *pellegrini* on the other hand, behaves more like an active forager preying on more diverse preys, although it can also feed on flying prey, which are typically consumed by sit and wait foragers. Our initial expectations were not met regarding the potential trophic niche overlap between *P. vaucheri* and *pellegrini*. Against our expectations, the diet composition of *P. vaucheri*, was found to be more similar to the diet of *chabanaudi*, regardless of its striped body pattern. The similar pigmentation of *P. vaucheri* and *pellegrini* plus their predisposition to live in the same type of habitats (Schleich et al., 1996), might increase interspecific competition. Therefore, adopting distinct foraging strategies reducing their niche overlap can help to reduce competition. However, due to small sample size, conclusions about *P. vaucheri* foraging behaviour should be made with caution.

Conclusions

Scelarcis perspicillata is a species complex (Harris et al., 2003) and the present study corroborates that different *S. perspicillata* forms adopt different dietary habits when living in syntopy. Huey and Pianka (1981) found that the diet of widely foraging lizards overlap more with other widely foraging lizards than they do with sit and wait foragers living in the same habitat. The 'niche overlap theory' suggests that the stronger competition there is, the less niche overlap. The discrimination between these two modes by *chabanaudi* and *pellegrini* might be an adaptation through decreasing trophic niche overlap to reduce intraspecific competition in that specific location, and thus allowing its coexistence in syntopy.

Metabarcoding can be an easy and quick method to describe diet composition and to complement already existing information thanks to its finer sensitivity and higher performance in cases of prey difficult to detect using microscopy. When field studies regarding foraging modes are not conceivable due to the inaccessibility of sampling areas or difficulty to visually identify prey (Kartzinel & Pringle, 2015), studies on diet composition might be an alternative whenever other species specific traits are well known (such as morphology). Nevertheless, the impossibility to compare size and hardness of eaten preys when using metabarcoding might be limiting, since these traits

appear to have a major importance in lizards' prey selection (e.g. Díaz & Carrascal, 1990). However, all these setbacks are likely to be overcome in the near future as molecular databases of biodiversity continue to grow, and barcodes will allow identification to higher taxonomic levels.

In this study we also conclude that primer choice can constitute a methodological constraint as some taxa are prevented from amplification. For example, the detection of Aranea in this study was only verified when using COI primers. This arthropod group seems to have a relatively high incidence in the diet of the studied species (Perera et al., 2006). However, we could not measure its frequency of occurrence in the sampled individuals due to the low specificity of COI marker. This highlights the appropriateness of the 16S marker for metabarcoding, but also the importance of using additional markers.

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3.2. Manuscript II

Ontogenetic and sexual differences in the diet of *Atlantolacerta andreanskyi* using DNA metabarcoding approaches

Abstract

Metabarcoding is being widely used in dietary studies allowing the detection of organisms present in mixed samples with a fine accuracy. It relies on the amplification of a group of interest using markers that should have appropriate taxonomic coverage and resolution in order to detect all present taxa. However, several biases can arise when using this methodology, such as PCR primer-template mismatches or the incomplete public databases for particular genomic regions to compare amplified sequences to. Nevertheless, this method can provide a non-invasive approach when studying diets, due to the possibility to reliably use faecal samples as starting material. *Atlantolacerta andreanskyi* is a small lacertid living in high altitudes of the high Atlas Mountains, Morocco, and it is thought to constitute a species complex. Although some populations present sexual dimorphism, diet was analysed in a population with insignificant dimorphism, using COI and 16S rRNA markers. Diet had previously been assessed using microscopy, so results could be compared between methodologies and between markers. Results showed this is an insectivore species with minimal differences between the diets of males and females. Additional prey items from the Acrididae (grasshoppers) and Formicidae (ants) families were identified relative to previous studies employing microscopy. Although the diet of females and males was similar, there were important differences between adults and juveniles, with the latter consuming less prey diversity with higher prevalence of Hemiptera. The large amount of non-target sequenced OTUs with COI marker highlighted the importance of primers choice, and the value of using multiple barcodes.

Introduction

Understanding the role of consumer-resource interactions in ecological communities faces the challenge of accurately identify what is being eaten, especially when studying generalist predators (DeBarba et al., 2013; Pompanon et al., 2012), such as lizards (Díaz & Carrascal, 1990). Molecular tools provide the possibility to identify multiple species from degraded environmental samples via high-throughput sequencing (Taberlet et al., 2012). DNA metabarcoding is being increasingly applied to dietary

studies to identify consumed organisms present in faecal or stomach samples (e.g. Sousa et al., 2016; Jarman et al., 2013; Shezhad et al., 2012). This method has the great advantage of allowing the analysis from one or more populations simultaneously by pooling hundreds of amplicons from different samples in a single mixture, and sequencing them in the same run (Pompanon et al., 2012). Since organisms present in small amounts can be detected (Kelly et al., 2014), this method provides a powerful tool to improve the level of information regarding the diets of individuals and/or populations.

All organisms must acquire sufficient energy to respond to their growth, reproduction and maintenance requirements. Therefore, an individual is expected to select among the available resources in order to maximize the rate of energy income (Bolnick et al., 2003). For some lizard species, variation in diet composition was shown to simply reflect differences on prey availability (Pérez-Mellado et al., 1991), and they are thought to be opportunistic feeders that prey upon what is available in the environment (Díaz & Carrascal, 1990). On the other hand, some authors concluded generalist predators rarely feed randomly on preys even if they are quite abundant (e.g. Symondson et al., 2002). Instead, prey choice seems to be more affected by prey defence mechanisms, energetic content and distribution, and intraspecific competition (reviewed in Symondson et al., 2002).

Several species undergo ontogenetic morphological changes, such as colour and body pigmentation (Booth, 1990). One of the best known adaptive coloration changes is the blue tail in juvenile lizards (Cooper & Vitt, 1985). It was hypothesized that blue tails might draw the attention of predators to attack that part the body enabling the lizard hatchling to autotomize the tail and escape (Booth, 1990). In fact, lizards having blue tails are more active in the field than others that have already undergone the ontogenetic colour change (Hawlena et al., 2006). Theoretically, active foraging behaviours, in this case by juveniles, is likely to result in the predation of less mobile and unpredictable prey (Huey & Pianka, 1981). The lacertid *Atlantolacerta andreanskyi* is a good example of this kind of ontogenetic shift in coloration where juveniles present a bright bluish green tail, which turns to brown in adults (Schleich et al., 1996). Colour change from juvenile to adult is also believed to be a mechanism of niche partitioning, helping to avoid intraspecific aggression and competition as adults can easily recognize juveniles by their specific colour patterns (Booth, 1990).

Ontogenetic shifts in diet are also widely recognized in reptiles, as in the case of the snake *Coronella austriaca*, that changes its preference from lacertids to small

mammals as it gets bigger (Reading & Jofré, 2013), and the cases of the freshwater turtle *Trachemys scripta* and some iguana species, such as *Ctenosaura pectinate*, that switch from a carnivorous and insectivorous diet, respectively, to herbivory as they grow (Bouchard & Bjorndal, 2005; Durtsche, 2000). These dietary shifts are thought to be related with the high nutritional properties of insects, providing the juveniles the needed resources for efficient growth (Durtsche, 2000). In the case of lacertid lizards, it is known that most species are largely insectivorous (VanDamme, 1999), and the most common ontogenetic differences are related to prey size, wherein juveniles eat smaller items than adults, which can prey upon both larger or smaller insects (Carretero et al., 2004). Lower prey diversity in juveniles when compared with adults is also an important ontogenetic dietary difference (Pérez-Mellado et al., 1991). For these reasons, when describing the ecology of a population, an individual perspective should be taken into account since individuals' resource use drive all types of social interactions, as intraspecific competition and response to predation (Bolnick et al., 2003).

Species living in harsh environments, such as alpine areas, are in general difficult to observe and sample, since reaching these areas is not easy and these species have frequently a protection status which limits handling. In these cases, using faecal sampling to study diets is essential since they can easily be obtained with minimum interaction and harm to animals (Pompanon et al., 2012). *Atlantolacerta andreanskyi* (Werner, 1929), commonly known as the Atlas dwarf lizard, is a lacertid lizard endemic to the High Atlas Mountains of Morocco, classified by the IUCN as Near Threatened. It is restricted to high altitudes, inhabiting areas between 2400 and 3800 m.a.s.l. (Bons & Geniez, 1996; Schleich et al., 1996). Individuals from this species have a preference for habitats close to small watercourses and thorny cushion plants as they are rich in food and at the same time work as shelter from predators and harsh weather (Schleich et al., 1996). The climate of mountain areas implies a long hibernation period for *A. andreanskyi*, from October to March (Schleich et al., 1996). Due to its extreme genetic diversity and isolation among populations, *A. andreanskyi* is thought to be a cryptic species complex (Barata et al., 2012; 2015). To date, the only quantitative dietary study of this species was in Oukaïmeden, where results showed that *A. andreanskyi* diet was mainly composed by Hemiptera and small proportions of Coleoptera and Araneae (Carretero et al., 2006) with no observed differences between sexes.

To date, the only study regarding sexual dimorphism for *A. andreanskyi* revealed a complex pattern, since the degree of dimorphism varied among the populations analysed (Barata et al., 2015). Oukaïmeden was one of the populations that exhibited

less marked sexual dimorphism. Thus, a segregation in the diet or niche resources is less likely (Barata et al., 2015), according to the previous study (Carretero et al. 2006). The aim of our research is to complement already existing information about the diet of *A. andreanskyi*, with the use of newer metabarcoding approaches to confirm whether there are differences between the diet of males and females, and to assess for the first time ontogenetic changes in the diet of this species. We expect to find a higher prevalence of small Coleoptera and other small clumped prey in juveniles' diet (Huey & Pianka, 1981), as well as other ontogenetic differences in diet resulting from divergent feeding behaviours. To accomplish our goals, we used a combination of highly degenerated COI primers (Leray et al., 2013) known to amplify across all metazoan taxa, and a 16S primer pair with high taxonomic coverage through several insect orders (Diptera, Lepidoptera, Hemiptera, Orthoptera, Coleoptera, Hymenoptera, Isoptera and Phasmatodea) (Clarke et al., 2014), to further assess the value of using multiple barcodes.

Material and Methods

Sampling details

A total of 51 individuals of *A. andreanskyi* were collected in September 2016 in Oukaïmeden (Morocco). From these, 20 were juveniles, 16 were females and 15 were males. All lizards were captured by hand, mostly inactive due to the rainy weather. Faecal pellets were collected from each lizard and stored in ethanol; standard measurements of body length were taken and adult specimens were sexed (see chapter 2). Lizards were then released unharmed in the place of capture.

Metabarcoding Methods

(Detailed in chapter 2 - Material and Methods)

Results

Sequences analysis

Amplification with COI primers was successful for ca 57% of all samples, which included 9 juveniles, 11 adult females and 9 adult males; For the 16S 71% of samples were successfully amplified, from which 10 were juveniles, 13 were adult females and 13 adult males. Data treatment was separately processed for COI and 16S sequences. Sequencing resulted in 546,746 merged sequences for the COI marker, and 652,138 for 16S. For the COI marker, a total of 469 clustered OTUs were produced, wherein 227

(49%) were non-food items (detailed in Figure 1). Some of these non-food sequences were clearly lab contaminations, such as *Stegastes* sp. (fish) or *Sus scrofa* (wild boar), as well as parasites which are commonly found in faeces, such as Nematodes. Although snails (Mollusca) are known to be part of *A. andreanskyi* diet, we decided not to consider these items as prey due to the presence of a large number of apparently marine contaminations and the impossibility to tell these sequences apart from real Gastropoda prey sequences. This contamination most probably occurred during the cleaning steps

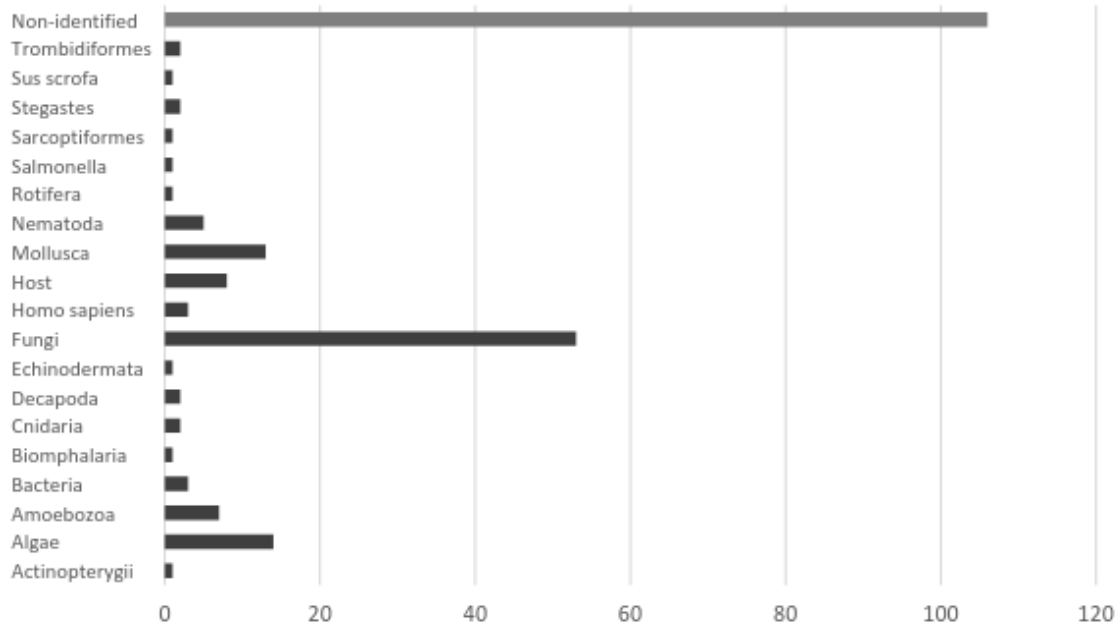

Figure 1 Number of non-food OTUs for COI gene.

when our PCRs were transferred and cleaned in plates shared with marine library samples of *Stegastes*' diet. Moreover, mites from the orders Trombidiformes and Sarcoptiformes, were neither considered since they could have been eaten accidentally or through secondary consumption. After removing non-food and contaminant OTUs from the dataset, a total of 242 OTUs were recovered, and the additional filtering based on the minimum number of OTUs considered per sample (minimum of 0.5 % of the total number retrieved for each sample, see Chapter 2 for details) resulted in 94 OTUs. A total of eight samples were exclusively constituted by non-food items and thus, removed from the dataset. For these reasons, COI sequences were not used in further comparative analysis, and used only to complement information attained with the 16S gene, in cases where new prey items were identified.

Regarding 16S, a total of 350 OTUs were clustered wherein two of them were lab contaminations and another one was identified as host DNA. From the remaining OTUs, 16 could not be taxonomically assigned. These 19 OTUs were removed from the

dataset. In addition, during the following filtering step sequences representing less than 0.5% of the total number retrieved for each sample were excluded. By the end, the dataset was restricted to 117 OTUs identified at least to the order level.

Diet composition

In general, prey items recovered belonged to the orders of Araneae, Coleoptera, Hemiptera, Isopoda, Hymenoptera, Lepidoptera and Orthoptera. Regarding adults, the most common prey were Coleoptera, followed by Hymenoptera, Orthoptera, Lepidoptera, Hemiptera, and Diptera. Interestingly, juveniles only predated upon Hemiptera, Coleoptera, and Hymenoptera. The difference in the number of consumed items is even more evident when prey were identified to the family level (Figure 2).

A total of 16 families were identified in the diet of adults and seven (i.e. less than 50%) in juveniles. Only 21% of the identified families were commonly present in both adults and juveniles. Moreover, prey frequency of occurrence shows they varied considerably between the two age classes, as for example in the case of Hemiptera, which is present in 50% of the individuals while in adults it was found in less than 5%.

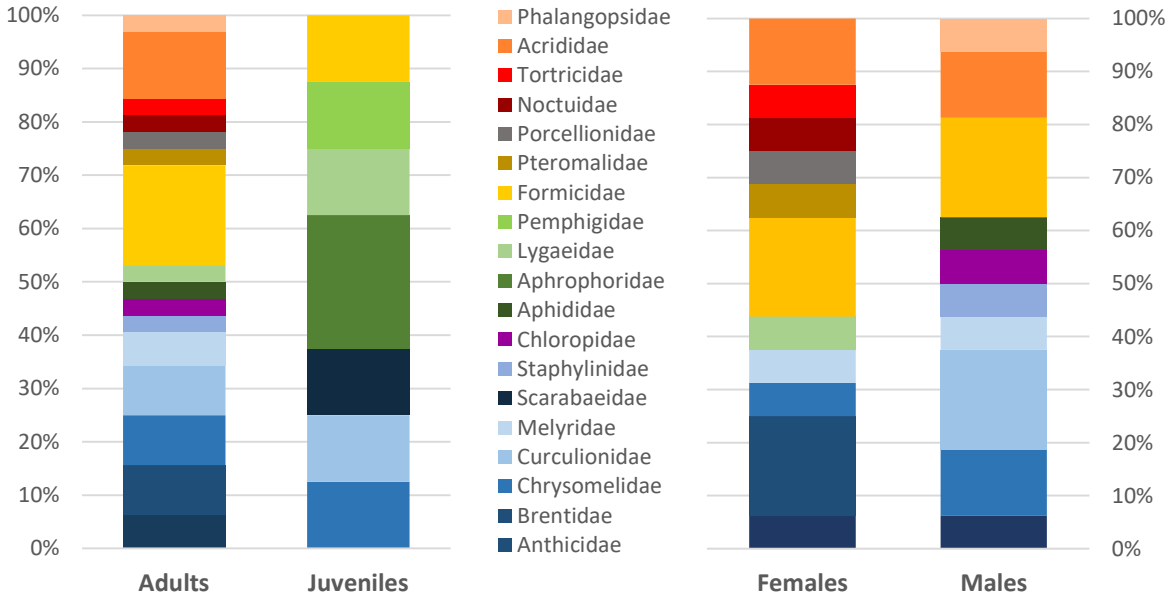

Figure 2 Frequency of occurence of prey items identified to the family level. Left side graphic shows differences between adults and juveniles, and in the right side differences between adult females and males are depicted. Families belonging to the Coleoptera order are represented in blue, Diptera in purple, Hemiptera in green, Hymenoptera in yellow, Isopoda in grey, Lepidoptera in red and Orthoptera in orange.

The number of predated families by females and males was similar, although only 29% of these families were common between them. The proportions of different

orders of prey are similarly distributed in males and females, despite differences regarding identified families. Among them, there is the exclusive presence of Lepidoptera and Isopoda in females, and Diptera in males.

Individual differences in diet

Our results showed high intraspecific variability in the diet, with a great variety in consumed items (Figure 3). Ants (hymenoptera from the family Formicidae) are the most frequent prey from all sampled lizards, but still present in only 7 individuals. On the other hand, flies (Chloropidae) were only found in one individual. The large difference in frequency of occurrence of Hemiptera (Fig. 3, coloured in green) and Coleoptera (Fig. 3, colored in blue) between juveniles and adults, respectively, is evident. Coleoptera species are usually characterized by having a hard exoskeleton and elytra (forewings), and therefore they are expected to require a longer handling time to be consumed than other prey of similar size (Díaz & Carrascal, 1990), and thus this might explain its higher consumption by adults. In juveniles, one to three prey items were found per individual,

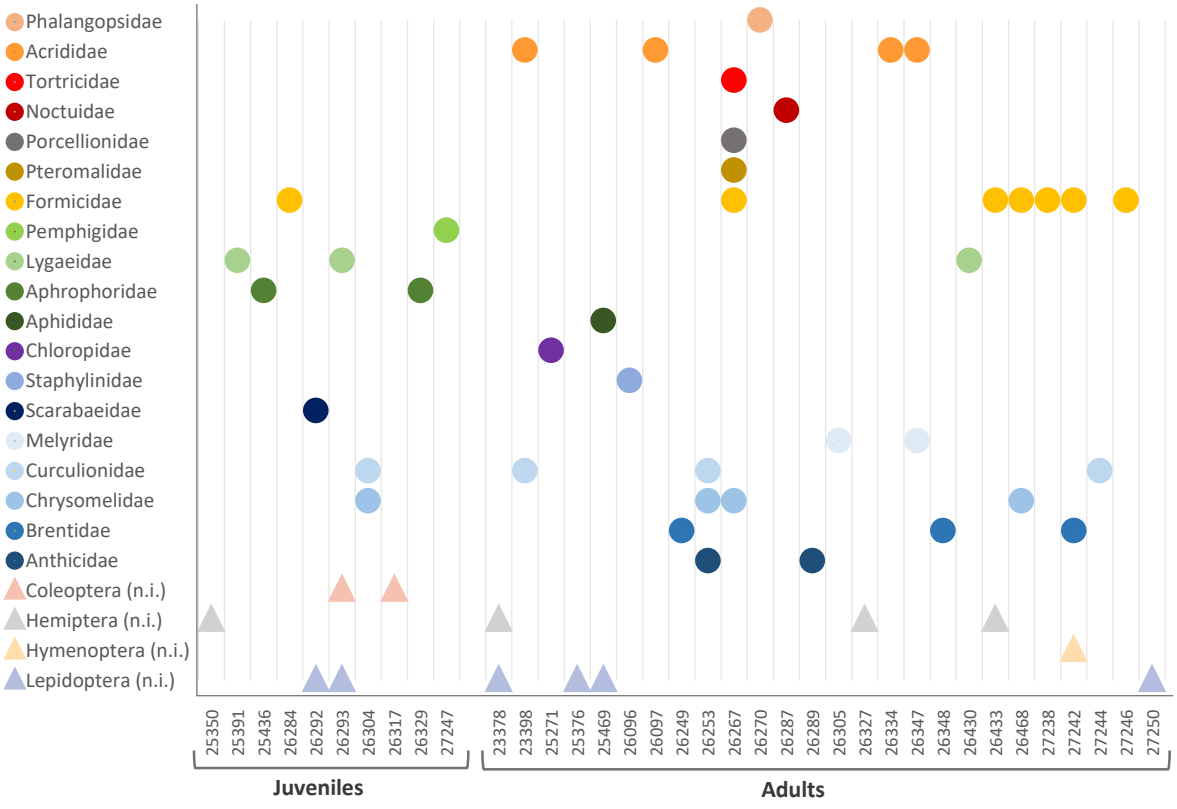

Figure 3 Number of occurrence of prey items per individual identified to the family level are depicted in circles. Prey items that could not be assigned to the family level are represented in triangles. Families are colored following Fig. 2.

however three different prey items were exclusively identified in one individual. Contrarily, up to five consumed preys were found in adult specimens, although some lizards had only consumed one prey item, or all items belonging the same order (e.g. Coleoptera).

Discussion

The results from the present study are similar to the previous work on the diet of *A. andreanskyi* in Oukaïmeden (Carretero et al., 2006), with the additional presence of two dietary items not described before. Moreover, it confirmed the expected higher diversity in the diet of adults and the consumption of a large proportion of soft bodied taxa by juveniles. Carretero et al. (2006) did not find Hymenoptera (Formicidae) or Orthoptera (Acrididae) in the diet of the population from Oukaïmeden. One possible reason for the differences found between the two studies might be the result of seasonality or interannual variation in invertebrates' availability. Carretero et al (2006) conducted the study with samples collected in April (Spring) of 2004, while our study was performed at the beginning of September (late Summer) of 2016. Interestingly, a decrease in the activity of ants is only expected during winter while in the warmer months, abundances are usually high (e.g. Kharbani & Hajong, 2013). The other hypothesis is that a metabarcoding approach allowed the detection of taxa that were not detected by microscopy and which therefore demonstrates the improved accuracy of the molecular approach.

The inclusion of individual differences as part of this study allowed a more complete description of the trophic interactions in this population. A pattern for preferred prey items is not evident across individuals and diet appears to be quite variable. For instance, one individual consumed prey belonging exclusively to Coleoptera while another one preyed on five different orders. Since conclusions made based on DNA barcoding are generally limited to presence/absence data, it is not possible to discriminate how many items were present in the diet. Instead, only frequency of different taxonomic units found in a sample can be assessed. A large proportion of Coleoptera was present in *A. andreanskyi* diet, and seven different families from this order could be identified, meaning that either DNA from these type of prey is more persistent after digestion being preserved in the hard parts, or Coleoptera items were in fact consumed with more frequency due to their high availability. A large consumption of Coleoptera can occur due to the adopted foraging behaviour, as this species is known to forage

intensively, examining holes and scratching hidden food items with the forelegs (Schleich et al., 1996).

Diet of males and females was considerably related, but with notable differences found at the family level, endorsing the advantages of metabarcoding over microscopy. Prey selection can be influenced by morphology, and for lacertids, specifically males select harder and bigger prey than females, which is positively correlated with head size and bite force capacity (Verwaijen et al., 2002). However, since the studied population presents low sexual dimorphism, small differences in selected prey between sexes were expected. At least in the Oukaïmeden population, this behaviour is probably adopted to reduce low levels of intraspecific competition, thus decreasing the need of diet specialization.

Smaller lizards are known to face higher predation risks than larger lizards due to the greater range of potential predators (Hawlena., 2009). This could mean the consumption of smaller, soft bodied prey in juveniles than in adults, due to the longer handling time required to consume harder prey, and thus longer exposure to predators. However, since hard bodied Coleoptera were also present in the diet of juveniles, we might hypothesize that juvenile lizards may carry the prey and consume them in safe places, as observed by Hawlena & Pérez-Mellado (2009). In fact, the consumption of highly energetic prey, such as Coleoptera by juveniles might be advantageous since there is a demand for rapid growth, particularly given their need to store reserves for the upcoming long hibernation period.

Considerations on marker choice

The high proportion of non-food items sequenced with the COI marker demonstrates the importance of primers choice. Other studies using the primer pair here used for diet studies (e.g. Leray et al., 2013) did not find as many contaminations as we did, probably due to the fact that previous studies used stomach contents, which seem to be less exposed to contamination by fungi and bacteria than pellets. Some studies may not report levels of contamination, on the supposition that if all contaminants are removed prior to analysis, they do not impact on results. However, we feel it is important to report contaminants to enable scientists to approach marker choice with as much information as possible. In our case, COI contamination was much more problematic than for 16S, which might indicate that the latter primers are more appropriate for this kind of dietary study of faecal pellets. On the other hand, the use of the COI marker was important to detect Araneae, which is not targeted by the 16S primers used in this study,

although more information regarding this prey item could not be obtained (such as frequency of occurrence). This highlights the advantages of using multiple markers in metabarcoding studies.

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4. General Discussion

The aim of this study was to use a metabarcoding approach to assess the dietary preferences of endemic lizards from Morocco. In the last decade, numerous genetic studies have identified the existence of cryptic diversity in Moroccan herpetofauna (e.g. Perera et al., 2007; Barata et al., 2015). In most of the cases, however, these cryptic species are still undescribed or waiting for further evidences (Barata et al., 2015) including, among other, ecological data. Complete diet profiles for those species might be complex to achieve, as traditional microscopic methods do not always allow the detection of all consumed taxa, especially in insectivorous consuming soft prey items with high digestibility. Given the appropriate marker and primer choice, metabarcoding has proved to constitute an effective method for detecting a wide range of taxa, including those with higher digestibility rates (e.g. Jarman et al., 2013). So far, one metabarcoding dietary research was published for lizards (Kartzinel & Pringle, 2015), and our studies confirm that metabarcoding can be a valuable complement to diet assessments in this group. The possibility of identifying prey items to the family level revealed extremely important regarding differences between the species herein studied. Our results are in accordance with what was published using microscopic identification, although they provide an extra layer of taxonomic resolution, by identifying preys to family level. We believe that the divergences identified could either be related to differences in prey availability between season and year of sampling (Pianka, 1973), or due to the methodological approach employed. Nevertheless, diet studies relying on prey identification from faecal samples constitute a snapshot of the last meal consumed if not assessed repeatedly over several years, and for this reason the results from this thesis are paramount.

4.1. Final considerations regarding marker choice

The use of two different markers in this study, the mitochondrial cytochrome oxidase 1 (COI) and the 16S rRNA, provided complementary information otherwise difficult to assess with a single marker. The high degeneracy and universality of COI primers led to the amplification of many contaminants and non-target taxa, which in turn reduced the overall sample size since many samples were exclusively constituted by non-target sequences. Previous studies using these primers for dietary assessments did not detect as many contaminations (Krehenwinkel et al., 2016; Leray et al., 2013; Leray et al., 2015), and I think this is due to differences related to the starting material for DNA extraction. While the other investigations relied on the amplification of dietary items from

gut samples, here faecal samples were used. Faeces are particularly enriched with fungi and bacteria, and I detected a massive amount of fungi reads that resulted in the removal of almost half of the produced sequences. The presence of large amounts of non-target amplicons has the added disadvantage of reducing sequencing depth for other prey items (Hajibabaei et al., 2011), which may have resulted in their elimination from the final dataset during data quality filtering. However, an exhaustive data quality filtering is of utmost importance when using next generation sequencing, to avoid contaminants in the final dataset. For that reason, I chose not compare diet diversity among samples for the COI marker. When testing the success of a PCR amplification through electrophoresis, the distinction between prey and non-target DNA is generally not possible. Therefore, the apparent amplification success of some samples can thus be an artefact and result in the exclusive sequencing of non-target items. Comparatively, the performance of the arthropod specific 16S primers allowed an almost exclusive detection of prey DNA, whereas most part of COI reads were identified as fungi, highlighting the importance of selecting group specific primers in dietary studies. Good performance of this gene in arthropod detection had already been pointed by Kartzinel & Pringle (2015), although the primer set used in our study revealed low affinity with some groups such as Araneae. The major drawback of using the 16S is that many sequences remained unidentified due to the low taxonomic coverage of the available databases. Therefore, the relevance of the marker choice, and the need of combining different markers are endorsed by this study.

4.2. Other technical limitations of metabarcoding

Although a metabarcoding approach may theoretically provide more accurate resolution on diet studies, there are several factors from sampling to bioinformatic analysis, that can challenge the reliability of a metabarcoding study and have to be considered. Besides the choice of the most appropriated primer set discussed above, the initial PCR step can influence the amplified taxa depending on the annealing temperature and number of cycles (Clarke et al, 2017). In this respect, the use of low annealing temperatures instead of a touchdown protocol might improve the amplification of additional taxa (Piñol et al., 2014). However, the use of predator blocking oligonucleotides requires high annealing temperatures at least in the beginning of the reaction, since these primers are normally longer than the ones used to amplify target taxa. So, although the use of blocking primers might enhance the probability of amplifying prey DNA, it may also prevent it (Vestheim & Jarman, 2008). Despite the fact that

blocking primers are currently the most used technique to remove predator DNA (e.g. Shehzad et al., 2012; Sousa et al., 2016), there are other options available. As prey DNA is expected to be more fragmented than predator DNA due to the digestion process, removal of high molecular weight DNA (using beads or gel excision, for example) could be one alternative to the use of blocking primers (Krehenwinkel et al., 2016).

Moreover, the choice for a dual-indexing strategy used in our study, that relies on the performance of two PCR amplifications might increase the well-known PCR biases. Nevertheless, the library preparation in a single step would have a considerable increased cost. Other laboratory steps have the potential to produce biased results. The use of magnetic beads to purify PCR products can wash away target DNA if the ratio of beads and PCR product is not accurately selected. However, if the right proportions are selected, purification with beads are widely used with no apparent constraints (e.g. Lundberg et al., 2013; Krehenwinkel et al., 2016; McInnes et al., 2017).

Despite the acknowledged high sensitivity of metabarcoding regarding species detection, implicit stochastic processes when amplifying samples with small amounts of starting material might also be a limitation (Ficetola et al., 2015). With the known high sensitivity to contamination of PCR protocols, a careful interpretation of unexpected taxa should be made (Yu et al., 2012). A large number of replicates, both for PCR and sequencing, can improve the confidence in our results by helping dealing with errors and reducing the risk of losing present taxa (e.g. Shezhad et al., 2012). However, the associated costs are much higher. In this study although three different PCR amplifications per sample were performed to avoid PCR biases, these were pooled prior to sequencing to allow maximizing the number of samples sequenced in a single MiSeq run (including other libraries).

The arbitrary choice of thresholds to remove low frequency sequences throughout the entire dataset to eliminate contaminants, can create the risk of discarding rare food items or prevent the detection of fine scale differences in diet (Pompanon et al., 2012). The use of a mock community of known prey items to help to select an appropriate threshold would be a good solution and is also a recommended procedure in order to confirm the reliability of the markers (Kozich et al., 2013). These data could further be used to assess the potential biases during bioinformatic data processing (Galan et al., 2017). Similarly, the creation of a reference database including all potential arthropods available in the habitat can also undoubtedly improve identification of the OTUs obtained in dietary assessments.

All these abovementioned technical aspects shape the results obtained. For this reason, standardizing metabarcoding analysis protocols would be essential in order to allow the validation of data quality and thus, to ensure accuracy and reliability of the results (DeBarba et al., 2013).

Metabarcoding has shown to be an easy and quick method in dietary studies, complementing information obtained with traditional methods due to its capacity to detect different types of prey. When field studies regarding foraging modes are not conceivable due to the inaccessibility of the study areas or other restricting factors, metabarcoding can constitute an alternative approach to infer those type of behaviours based on lizards' consumed prey. Nevertheless, the impossibility of comparing prey sizes, hardness and number of items consumed when using metabarcoding is still an important limitation to this approach, since these characteristics were shown to have major influence in understanding lizards' prey selection and feeding strategies (e.g. Díaz & Carrascal, 1990). Only morphological examination of pellets or stomach/gut contents can efficiently distinguish between different life stages of several prey taxa, such as the ones that undergo metamorphosis. The detection of larvae prey might be essential to infer foraging modes as only active foragers are expected to find sedentary or cryptic prey (Verwaijen & Van Damme, 2007). Furthermore, the importance of larvae consumption by juveniles should be evaluated since ontogenetic differences in prey digestion might reflect differences in energy and nutrients assimilation (e.g. Bouchard & Bjorndal, 2006). Therefore, the exclusive use of metabarcoding when relying on diet to infer ecological processes might be insufficient since other traits rather than prey taxonomy are relevant. Contrasting conventional barcoding, species-level identification might be unnecessary in some metabarcoding applications, as the case of diet studies. Identification to reasonably high taxonomic levels, such as family or order, are often sufficient (Coissac et al., 2012).

Future investigations using metabarcoding to assess dietary profiles of lizards using faecal pellets should have into consideration the selected sample size, both bench and bioinformatic. All the molecular procedures might lead to removal of part of the dataset due to failed PCR amplifications, low quality sequences or even contaminations. In this respect, we consider our sample size was not enough as a result of these unexpected issues. Moreover, we consider fundamental to collect as many pellets as possible for each sample in order to increase the detection of more taxa.

4.3. *Scelarcis perspicillata* and *Atlantolacerta andreanskyi*: Future research perspectives

Differences found between this study and previous studies can be related to several biological and methodological factors, such as the fluctuation of prey abundances and diversity between seasons, sample size, different methodologies to detect prey, or simply differences on the last meal taken by the sampled lizards. Therefore, it would be important to compare the diet composition using both metabarcoding and morphology using the same samples so that the best of the two methods can be used to complement each other and provide a more complete picture. Moreover, a long term study throughout different seasons and surveying the insect availability in the community would reduce big part of the present study uncertainties.

Finally, both ours and previous studies are punctual allowing only to retrieve a snapshot of the actual diet of the lizards. Thus, integrating microscopy, metabarcoding and isotope analysis, for instance, would help to clarify patterns related to foraging modes and diet partitioning within populations.

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Supplementary Material

Table S1 – List of tested primers.

Name	Marker	Sequence (5'-3')	Length (bp)	Reference
Ins16S_1	16S	TRRGACGAGAAGACCCTATA/ TCTTAATCCAACATCGAGGTC	216	Clarke et al., 2014
Ins16S_1short	16S	TRRGACGAGAAGACCCTATA/ ACGCTGTTATCCCTAAGGTA	156	Clarke et al., 2014
Ins16S_9	16S	GATAGAAACCAACCTGGCT/ AARTTACCTTAGGGATAACAGC	141	Clarke et al., 2014
coleop_16Sc/ coleop_16Sd	16S	TGCAAAGGTAGCATAATMATTAG/ TCCATAGGGTCTTCTCGTC	107	Epp et al., 2012
IN16STK-1F/ IN16STK-1R	16S	TGAACTCAGATCATGTAA/ TTAGGGATAACAGCGTAA	107	Kartzinel et al., 2015
LepF1/ EPT-long-univR	COI	ATTCAACCAATCATAAAGATATTGG/ AARAAAATYATAAYAAAIGCGTGIAIIGT	130	Hajibabaei et al., 2011
Ins3R/Ins3L	COI	TCCTGTTGGAACAGCAATAAT/ AAAGAAACATTTGGAGCTTTAGGA	114	Thomsen et al., 2009
Uni-MinibarF1/ Uni-MinibarR1	COI	TCCACTAATCACAARGATATTGGTAC/ GAAAATCATAATGAAGGCATGAGC	127	Meusnier et al., 2008
ZBJ-ArtF1c/ ZBJ-ArtR2c	COI	AGATATTGGAACWTTATATTTTATTTTTGG/ WACTAATCAATTWCCAAATCCTCC	157	Zeale et al., 2011
Teph227-L499/ Teph227-H2123d	COI	ATTAATATACGATCAACAGGAAT/ TAWACTTCWGGRTGWCCAAARAATCA	178	Van Houdt et al., 2009
mlCOIintF/ jgHCO2198	COI	GGWACWGGWTGAACWGTWTAYCCYCC/ TAIACYTCIGGRTGICCRAARAAYCA	313	Leray et al., 2013/ Geller et al., 2013

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Table S2 – List of samples amplified for this study.

COI					16S				
Sample ID	**Species**	**Sex**	**Age**	**SVL**	**Sample ID**	**Species**	**Sex**	**Age**	**SVL**
26334	*A. andreanskyi*	Female	Adult	39,01	23378	*A. andreanskyi*	Female	Adult	35,64
26305	*A. andreanskyi*	Female	Adult	39,43	26334	*A. andreanskyi*	Female	Adult	39,01
27242	*A. andreanskyi*	Female	Adult	41,03	26305	*A. andreanskyi*	Female	Adult	39,43
26097	*A. andreanskyi*	Female	Adult	42,86	26348	*A. andreanskyi*	Female	Adult	40,54
26249	*A. andreanskyi*	Female	Adult	42,96	25376	*A. andreanskyi*	Female	Adult	40,63
26267	*A. andreanskyi*	Female	Adult	43,10	27242	*A. andreanskyi*	Female	Adult	41,03
26430	*A. andreanskyi*	Female	Adult	44,93	27246	*A. andreanskyi*	Female	Adult	41,77
26289	*A. andreanskyi*	Female	Adult	45,90	26287	*A. andreanskyi*	Female	Adult	41,88
27244	*A. andreanskyi*	Male	Adult	41,53	26097	*A. andreanskyi*	Female	Adult	42,86
26327	*A. andreanskyi*	Male	Adult	42,28	26249	*A. andreanskyi*	Female	Adult	42,96
26253	*A. andreanskyi*	Male	Adult	43,31	26267	*A. andreanskyi*	Female	Adult	43,10
26347	*A. andreanskyi*	Male	Adult	43,44	26430	*A. andreanskyi*	Female	Adult	44,93
23398	*A. andreanskyi*	Male	Adult	44,32	26289	*A. andreanskyi*	Female	Adult	45,90
27250	*A. andreanskyi*	Male	Adult	46,57	25271	*A. andreanskyi*	Male	Adult	39,23
26468	*A. andreanskyi*	Male	Adult	47,58	26433	*A. andreanskyi*	Male	Adult	39,52
23364	*A. andreanskyi*	-	Juvenile	23,03	27244	*A. andreanskyi*	Male	Adult	41,53
26317	*A. andreanskyi*	-	Juvenile	28,82	26327	*A. andreanskyi*	Male	Adult	42,28
26329	*A. andreanskyi*	-	Juvenile	30,02	25469	*A. andreanskyi*	Male	Adult	42,54
26293	*A. andreanskyi*	-	Juvenile	31,32	26253	*A. andreanskyi*	Male	Adult	43,31
26292	*A. andreanskyi*	-	Juvenile	32,68	26270	*A. andreanskyi*	Male	Adult	43,44
26304	*A. andreanskyi*	-	Juvenile	34,25	26347	*A. andreanskyi*	Male	Adult	43,44
26054	*P. vaucheri*	Male	Adult	39,88	26096	*A. andreanskyi*	Male	Adult	43,71
27567	*P. vaucheri*	Male	Adult	40,41	27238	*A. andreanskyi*	Male	Adult	44,00
26326	*P. vaucheri*	Male	Adult	40,48	23398	*A. andreanskyi*	Male	Adult	44,32
27574	*P. vaucheri*	Male	Adult	41,03	27250	*A. andreanskyi*	Male	Adult	46,57
26424	*P. vaucheri*	Male	Adult	41,21	26468	*A. andreanskyi*	Male	Adult	47,58
25372	*P. vaucheri*	Male	Adult	42,15	26284	*A. andreanskyi*	-	Juvenile	28,78
27237	*P. vaucheri*	Male	Adult	42,70	26317	*A. andreanskyi*	-	Juvenile	28,82
27644	*P. vaucheri*	Male	Adult	44,30	26329	*A. andreanskyi*	-	Juvenile	30,02
27243	*P. vaucheri*	Male	Adult	45,16	26293	*A. andreanskyi*	-	Juvenile	31,32
27581	*P. vaucheri*	Male	Adult	47,49	26292	*A. andreanskyi*	-	Juvenile	32,68
26303	*P. vaucheri*	Male	Adult	48,90	25391	*A. andreanskyi*	-	Juvenile	33,31
27570	*P. vaucheri*	Male	Adult	52,46	25436	*A. andreanskyi*	-	Juvenile	33,96
27252	*P. vaucheri*	Male	Adult	53,81	26304	*A. andreanskyi*	-	Juvenile	34,25
26306	*S. p. chabanaudi*	Female	Adult	51,00	25350	*A. andreanskyi*	-	Juvenile	34,58
26291	*S. p. chabanaudi*	Female	Adult	51,74	27247	*A. andreanskyi*	-	Juvenile	34,70
26104	*S. p. chabanaudi*	Female	Adult	52,24	26326	*P. vaucheri*	Male	Adult	40,48
26321	*S. p. chabanaudi*	Female	Adult	58,45	27574	*P. vaucheri*	Male	Adult	41,03
25438	*S. p. chabanaudi*	Female	Adult	59,50	26424	*P. vaucheri*	Male	Adult	41,21
27253	*S. p. chabanaudi*	Female	Adult	60,98	25372	*P. vaucheri*	Male	Adult	42,15

25374	*S. p. chabanaudi*	Female	Adult	62,50
26405	*S. p. chabanaudi*	Female	Adult	62,64
27577	*S. p. chabanaudi*	Female	Adult	63,68
26294	*S. p. chabanaudi*	Female	Adult	64,50
27582	*S. p. chabanaudi*	Female	Adult	68,60
25389	*S. p. chabanaudi*	Male	Adult	59,89
26085	*S. p. chabanaudi*	Male	Adult	60,26
26309	*S. p. chabanaudi*	Male	Adult	61,96
27579	*S. p. pellegrini*	Female	Adult	41,60
27561	*S. p. pellegrini*	Female	Adult	43,97
23399	*S. p. pellegrini*	Female	Adult	44,13
23405	*S. p. pellegrini*	Female	Adult	44,52
27558	*S. p. pellegrini*	Female	Adult	44,70
27556	*S. p. pellegrini*	Female	Adult	46,67
26698	*S. p. pellegrini*	Female	Adult	46,74
27557	*S. p. pellegrini*	Female	Adult	47,25
27576	*S. p. pellegrini*	Female	Adult	49,41
23400	*S. p. pellegrini*	Female	Adult	50,55
27566	*S. p. pellegrini*	Female	Adult	50,89
26320	*S. p. pellegrini*	Male	Adult	47,53
27573	*S. p. pellegrini*	Male	Adult	50,05
27572	*S. p. pellegrini*	Male	Adult	51,00
27580	*S. p. pellegrini*	Male	Adult	51,55
23362	*S. p. pellegrini*	Male	Adult	51,79
27563	*S. p. pellegrini*	Male	Adult	52,78
27634	*S. p. pellegrini*	Male	Adult	54,20
27650	*S. p. pellegrini*	Male	Adult	54,78
27630	*S. p. pellegrini*	Male	Adult	55,00

27237	*P. vaucheri*	Male	Adult	42,70
26303	*P. vaucheri*	Male	Adult	48,90
27252	*P. vaucheri*	Male	Adult	53,81
26419	*S. p. chabanaudi*	Female	Adult	47,70
26306	*S. p. chabanaudi*	Female	Adult	51,00
26291	*S. p. chabanaudi*	Female	Adult	51,74
26104	*S. p. chabanaudi*	Female	Adult	52,24
26321	*S. p. chabanaudi*	Female	Adult	58,45
25438	*S. p. chabanaudi*	Female	Adult	59,50
27253	*S. p. chabanaudi*	Female	Adult	60,98
27251	*S. p. chabanaudi*	Female	Adult	61,50
27577	*S. p. chabanaudi*	Female	Adult	63,68
27582	*S. p. chabanaudi*	Female	Adult	68,60
26309	*S. p. chabanaudi*	Male	Adult	61,96
25461	*S. p. chabanaudi*	Male	Adult	68,42
27561	*S. p. pellegrini*	Female	Adult	43,97
27558	*S. p. pellegrini*	Female	Adult	44,70
27640	*S. p. pellegrini*	Female	Adult	46,35
27556	*S. p. pellegrini*	Female	Adult	46,67
27557	*S. p. pellegrini*	Female	Adult	47,25
27623	*S. p. pellegrini*	Female	Adult	47,31
27576	*S. p. pellegrini*	Female	Adult	49,41
27624	*S. p. pellegrini*	Female	Adult	51,67
26320	*S. p. pellegrini*	Male	Adult	47,53
27564	*S. p. pellegrini*	Male	Adult	48,00
27573	*S. p. pellegrini*	Male	Adult	50,05
27572	*S. p. pellegrini*	Male	Adult	51,00
27580	*S. p. pellegrini*	Male	Adult	51,55
27650	*S. p. pellegrini*	Male	Adult	54,78
27630	*S. p. pellegrini*	Male	Adult	55,00